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Doctor of Philosophy Degree
15 March 2006

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Sharon X. R. Wen

Department of Microbiology and Immunology

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Abstract

Title of Dissertation:

Multi-faceted Approach to Vaccine Development Against *Escherichia coli* O157:H7

Sharon X. R. Wen

Thesis directed by:

Alison O'Brien, Ph.D.

Professor and Chair, Department of Microbiology and Immunology

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 was first recognized as a human pathogen in the early 1980s when it was isolated from the stools of individuals with bloody diarrhea who had ingested undercooked hamburger. We now know that cattle are asymptomatic intestinal carriers of O157:H7 and the principal source of transmission to people. The primary adhesin of O157:H7, intimin, is required for the organism to colonize cattle. Additionally, EHEC can produce one or more types of potent Shiga toxins (Stx) that are responsible for the damage to renal microvasculature that can lead to the development of the life-threatening post-infection sequela, the hemolytic uremic syndrome (HUS). The goal of our research is to develop a vaccine against EHEC that can reduce transmission of the organism from cattle to humans and

also prevent toxin-mediated disease in infected individuals. Ideally, such a vaccine would induce mucosal anti-intimin IgA antibodies that block adherence of the bacteria to the gut and serum anti-Stx IgG antibodies that neutralize toxin activity. It was recently reported that intimin-expressing tobacco cells, when used in a parenteral-prime-oral-boost immunization scheme, reduced the colonization of O157:H7 in mice. Further efforts to express intimin in corn are described here as well as are my efforts to design and evaluate prototype immunogens against Stxs. For the latter purpose, I initially demonstrated that genetic toxoids are viable vaccine candidates, and confirmed the hypothesis that a multivalent vaccine for both Stx 1 and 2 is required. Next, I made a plant-based Stx2 toxoid vaccine, fed it to mice, and demonstrated that it protected them from intoxication after oral challenge with an Stx-producing *E. coli* strain. Taken together, these results, along with our previous findings, support the theory that a single plant-based, orally delivered, multivalent vaccine against EHEC composed of intimin and Stx1 and 2 toxoids could eventually be used in cattle to reduce transmission to people and in humans to prevent HUS. Alternatively, an intimin-based vaccine alone could be used in cattle and a separate toxoid-based plant vaccine could be given to people.

Multi-faceted Approach to Vaccine Development Against *Escherichia coli* O157:H7

By

Sharon Xiao Rong Wen

Dissertation submitted to the Faculty of the

Emerging Infectious Diseases Interdisciplinary Graduate Program of the

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F. Edward Hèbert School of Medicine

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy 2006

To all the little furry fellows in cages:

their sacrifices pave the way for advancements in science.

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To all my friends in USHUS: May our happy hours always be warm and fuzzy.

To Jon: Your friendship has made this journey a lot easier.

To my husband, Richard: We made it (high five)!

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Chapter One

Introduction

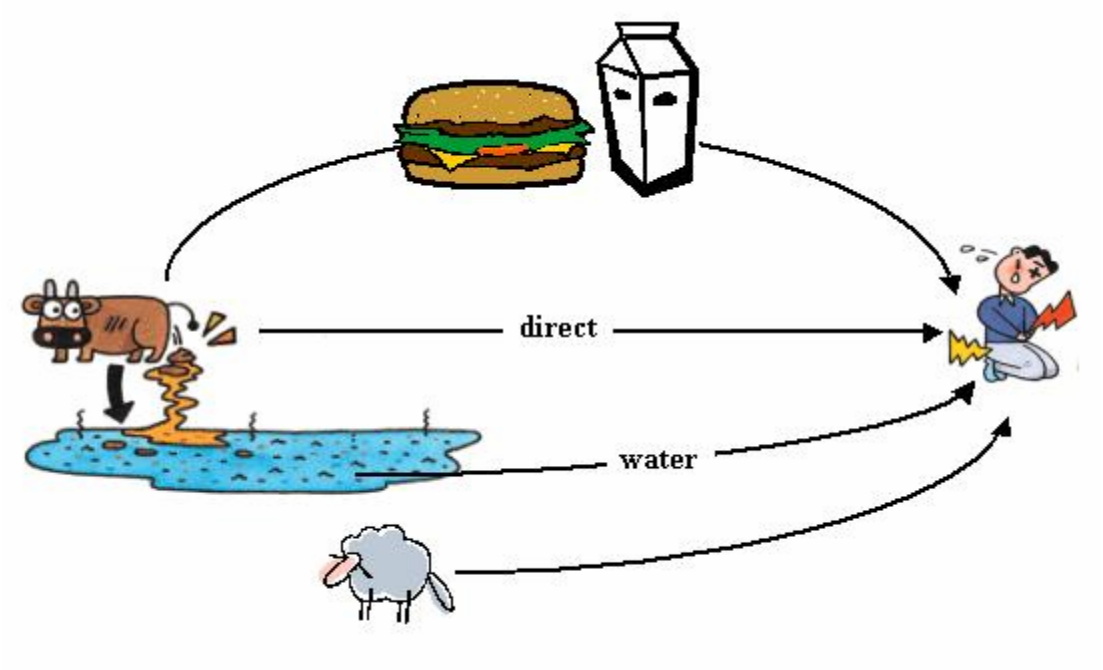
Preface

Escherichia coli (*E. coli*) O157:H7 is a representative pathotype of Enterohemorrhagic *E. coli* (EHEC) that has gained much prominence because of the distinct clinical features associated with EHEC infection, hemorrhagic colitis or bloody diarrhea, and the hemolytic uremic syndrome (HUS). This bacterium is also infamous because of its association with the ingestion of undercooked hamburgers. The emergence of this pathogen in the 1980s as a potentially life-threatening illness in otherwise healthy individuals, particularly young children, has led to a significant scientific effort to understand key characteristics of the microbe and its associated diseases (Fig. 1). Current research on *E. coli* O157:H7 is focused on identifying and analyzing the virulence determinants of the organism, analyzing its genome, designing methods to curtail transmission of the bacteria from farm to table, understanding the pathogenesis of the most serious aspect of EHEC O157:H7 infection, the HUS, and developing vaccines and therapeutics to prevent and treat *E. coli* O157:H7-mediated illness, respectively. The following introduction to this thesis is divided into several sections. First, an overview of *E. coli* O157:H7 is presented with emphasis on its discovery as a disease entity, types of illnesses it can cause, epidemiology, routes of transmission, natural reservoirs, pathogenic determinants, diagnosis, and current treatment modalities. Second, Shiga toxin (Stx), the major virulence factor of *E. coli* O157:H7 that is responsible for the more severe manifestations of disease, is described. Third, intimin, the primary adhesin of *E. coli* O157:H7 is discussed. In each of these latter sections, the potential for Stx toxoids and intimin as vaccine candidates is considered. Fourth, the strategy of using transgenic

plants to express foreign antigens as a means of producing orally-administered vaccines is reviewed. Finally, in the last section of the introduction, the hypothesis and specific aims of this dissertation are presented.

Figure 1. Overview of EHEC O157:H7 life cycle.

Cattle are the natural reservoir of EHEC O157:H7. The bacteria colonize the gastrointestinal tracts, surface hides, and oral cavities of cattle without causing any disease symptoms. This colonization contributes directly to contamination of food of bovine origin, such as beef and dairy products. The fecal shedding of the organism into the environment leads to contamination of water and crops and potentially increases the risk that other farm animals will become colonized with EHEC O157:H7. Direct transmission from colonized cattle or other farm animals such as sheep in petting zoos to humans can also occur, as well as human-to-human spread. The organism can cause mild to bloody diarrhea and, in a minority of patients, the HUS.


direct
water

Enterohemorrhagic *E. coli* O157:H7

History

The first documented report of *E. coli* O157:H7 isolation in the United States was in 1982 from a single-source outbreak of acute gastrointestinal illness that occurred in Oregon and Michigan and was linked to the same fast-food restaurant chain (Center for Disease Control 1982). The illness in affected individuals was characterized by severe abdominal cramps and bloody diarrhea (Center for Disease Control, 1982). As the result of the intensive investigation that followed the outbreak, *E. coli* serotype O157:H7 was identified for the first time by Riley *et al*. as a human pathogen (Riley *et al*., 1983). Riley and his colleagues also made the association between ingestion of hamburger, possibly undercooked, and development of hemorrhagic colitis (Riley *et al*., 1983). In spite of these seminal observations, *E. coli* O157:H7 remained relatively unknown as a pathogen for the next 10 years, probably because, on average, less than 3 outbreaks were reported per year (Rangel *et al*., 2005a). However, in 1993, a large (> 500 individuals) multistate outbreak of *E. coli* O157:H7 disease occurred in the United States that featured not only hemorrhagic colitis but also HUS and again implicated undercooked ground beef patties as the cause of the infection (Center for Disease Control 1993). At this time, *E. coli* O157:H7 gained attention as an important food-borne pathogen (Bell *et al*., 1994).

Diseases

Clinical manifestations of EHEC O157:H7 infection range from none (asymptomatic carriage) to non-bloody diarrhea, to bloody diarrhea, to the hemolytic uremic syndrome (Fig. 2). The average interval between exposure and onset of disease is 3-4 days (Mead

et al., 1999) The infected person usually presents with little or no fever and recovers in 5 to 10 days. In some patients, especially children under 5 years of age and the elderly, the infection can also cause a potentially life-threatening complication called the hemolytic uremic syndrome (HUS) that is characterized by thrombocytopenia, hemolytic anemia, and renal failure. About 5% of infections in adults, and as high as 15% in children, can lead to this sequela. In the United States, EHEC O157-mediated HUS is the principal cause of acute kidney failure in children (Center for Disease Control 1994).

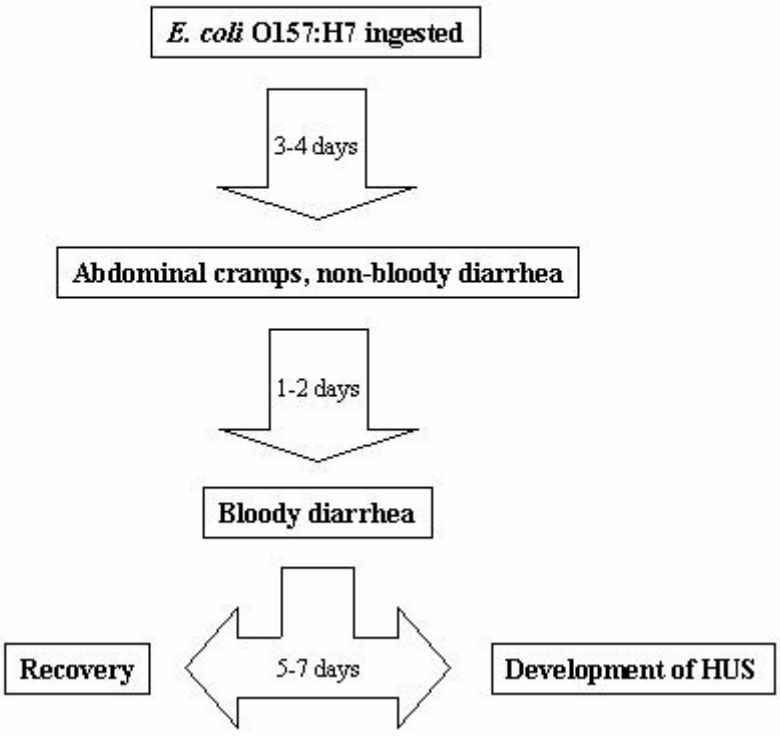
Epidemiology

In response to the outbreaks in the early 1990s, clinical laboratories began examining more stool samples, especially bloody specimens, for *E. coli* O157:H7 (Boyce *et al*., 1995). In 1994, *E. coli* O157:H7 became a nationally notifiable infection. In 1996, *E. coli* O157:H7 was one of the pathogens for which that the Foodborne Diseases Active Surveillance Network (FoodNet) began active, population-based surveillance. Then in 1997, *E. coli* O157:H7 was included in the national network for detecting common foodborne disease case clusters by pulsed-field gel electrophoresis (PulseNet). By 2000, reporting of cases of *E. coli* O157:H7 infection was mandatory in 48 states. The implementation of these surveillance systems coincided with a sharp increase in reported incidents of *E. coli* O157 outbreak as illustrated in figure 3. In 1999, Mead *et al*. estimated that in the United States, *E. coli* O157 causes an estimated 73,480 cases of food-borne illness, leading to an estimated 2,168 hospital stays and 61 deaths annually (Mead *et al*., 1999). These data may now be an overestimate of the incidence and, in fact

Figure 2. Disease outcome from EHEC O157:H7 infection .

The incubation period is usually 3-4 days. Illness typically begins with severe abdominal cramps and non-bloody diarrhea. Stool specimens may become bloody over the next 1-2 days with the amount of visible blood ranging from small streaks to significantly bloody. Most patients with hemorrhagic colitis recover spontaneously within 7 days. Approximately 5% of patients develop HUS. Adapted from Mead *et al*., 1998.

Disease process with *E. coli* O157:H7 infection



there has been a decrease in the median outbreak size over time (Fig. 3). Nevertheless, currently, about forty percent (Tserenpuntsag *et al*., 2005) of patients with *E. coli* O157:H7 infection are hospitalized. The attendant medical expenses from such hospitalization as well as the cost of the lost productivity for adults who are ill or whose children are sick (or are forbidden from returning to day care without culture-negative stools) likely remains in the hundreds of millions annually [estimated at $200-600 million in 1996.(Buzby JC *et al*., 1996)].

EHEC O157:H7 is a major food-related health concern in the developed countries including the U.S., Canada, the United Kingdom, and Japan (Kaper *et al*., 1998b), and it is the leading cause of bloody diarrhea in the U.S. In a multicenter study by Slutsker *et al*., the rate of EHEC O157:H7 isolated from bloody stool samples collected around the country was higher than that of *Campylobacter*, *Salmonella*, or *Shigella* (Slutsker *et al*., 1997). Between 1991 and 1996, 30+ outbreaks of EHEC O157:H7 infection were reported in Japan, including several major outbreaks in 1996 that affected more than 5000 school children (Takeda 1997). In 1996, central Scotland reported an EHEC O157:H7 outbreak that involved more than 500 people and killed 20 of those infected individuals (Ahmed *et al*., 1998). Incidents of O157:H7 isolation are also on the rise in South America, especially in Argentina where the exceptionally high frequency of HUS in children is explained by the high incidence of EHEC-mediated bloody diarrhea (Lopez *et al*., 1998).

Transmission

Since its national debut in 1982, *E. coli* O157:H7 has received extensive media coverage as an emerging infectious pathogen that causes sporadic outbreaks even when only a low dose of the bacteria is found in the implicated food source (Tilden, Jr. *et al*., 1996). The initial association of *E. coli* O157:H7 with undercooked beef patties frozen and sold in bulk to grocery stores or fast-food restaurants earned the organism the nickname "the hamburger bug" (FSNet 1998). However, a review by Rangel *et al*., of epidemiological studies done during and following outbreaks of *E. coli* O157 in this country between 1982-2002 revealed many other modes of transmission of the organism (Rangel *et al*., 2005a) (Fig. 4). These routes of spread can be categorized into foodborne, person-to-person, waterborne, animal-to-person, laboratory-related, or unknown.
In the foodborne category, undercooked ground beef in hamburgers is the most common vehicle for *E. coli* O157. The peak incidence for disease associated with these patties is in the summer months (Rasmussen *et al*., 2001) when many individuals grill outdoors. Other types of beef, such as raw roast beef or steaks, have also been implicated in some sporadic clusters of *E. coli* O157disease (Rangel *et al*., 2005a). Dairy products associated with raw milk are another source of contamination that can lead to community-wide outbreaks (Rangel *et al*., 2005a). In addition to foods of bovine origin, produce such as lettuce, apple cider, melons and sprouts have also been incriminated as the vehicle of EHEC O157:H7 transmission in several outbreaks (Mermin *et al*., 1999). The incidence of cases of produce-associated of *E. coli* O157-mediated disease, like the frequency of illness where ground beef is the source, also peaks in the summer months

Figure 3. Number and median size of EHEC O157:H7 outbreaks, 1982 to 2002.

A total of 350 outbreaks of *E. coli* O157 were reported from 1982 to 2002, with a sharp increase after 1992 and a peak in 2000. The median size of each reported outbreak, however, declined over the years (Rangel *et al*., 2005a).

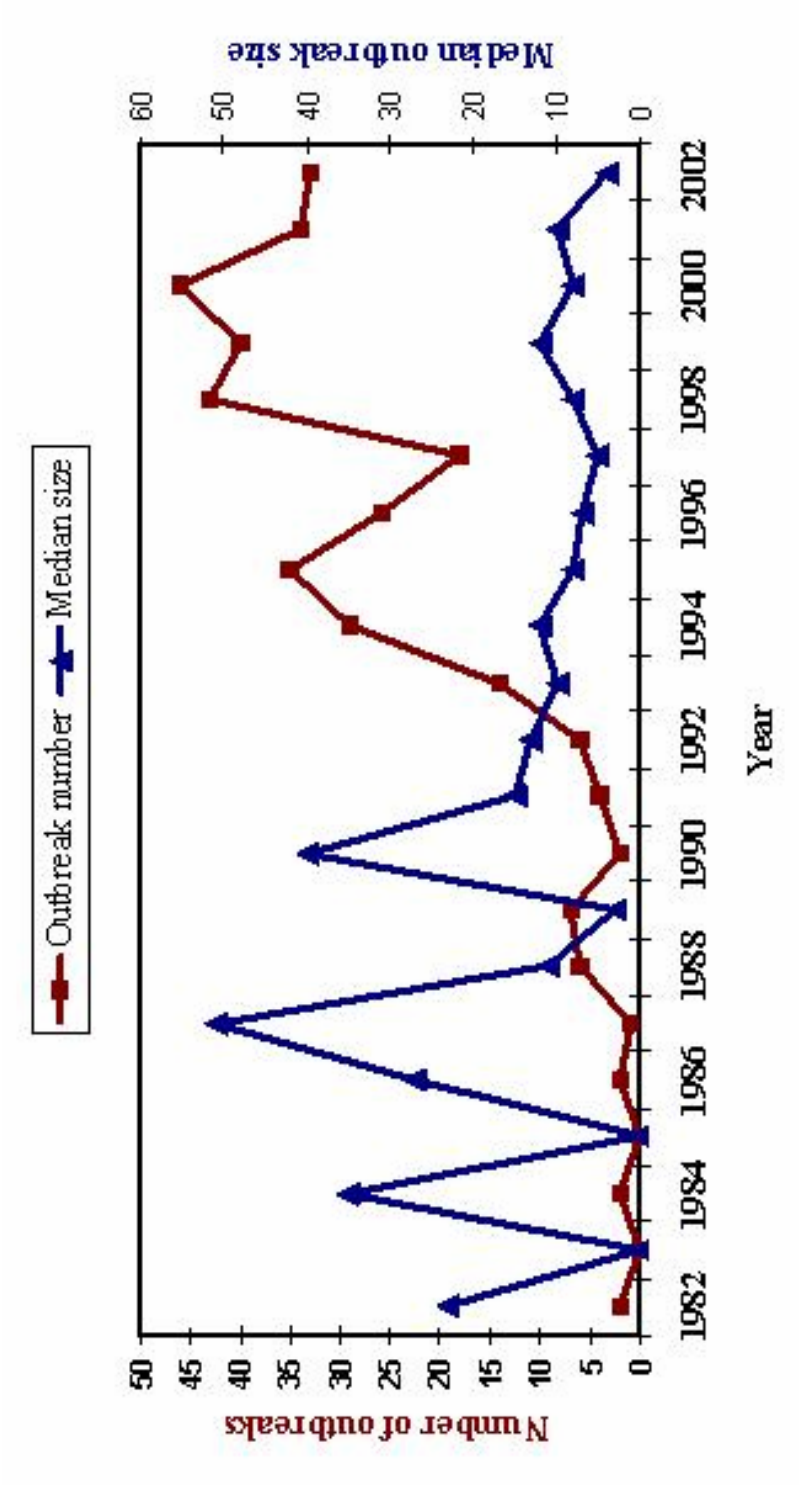
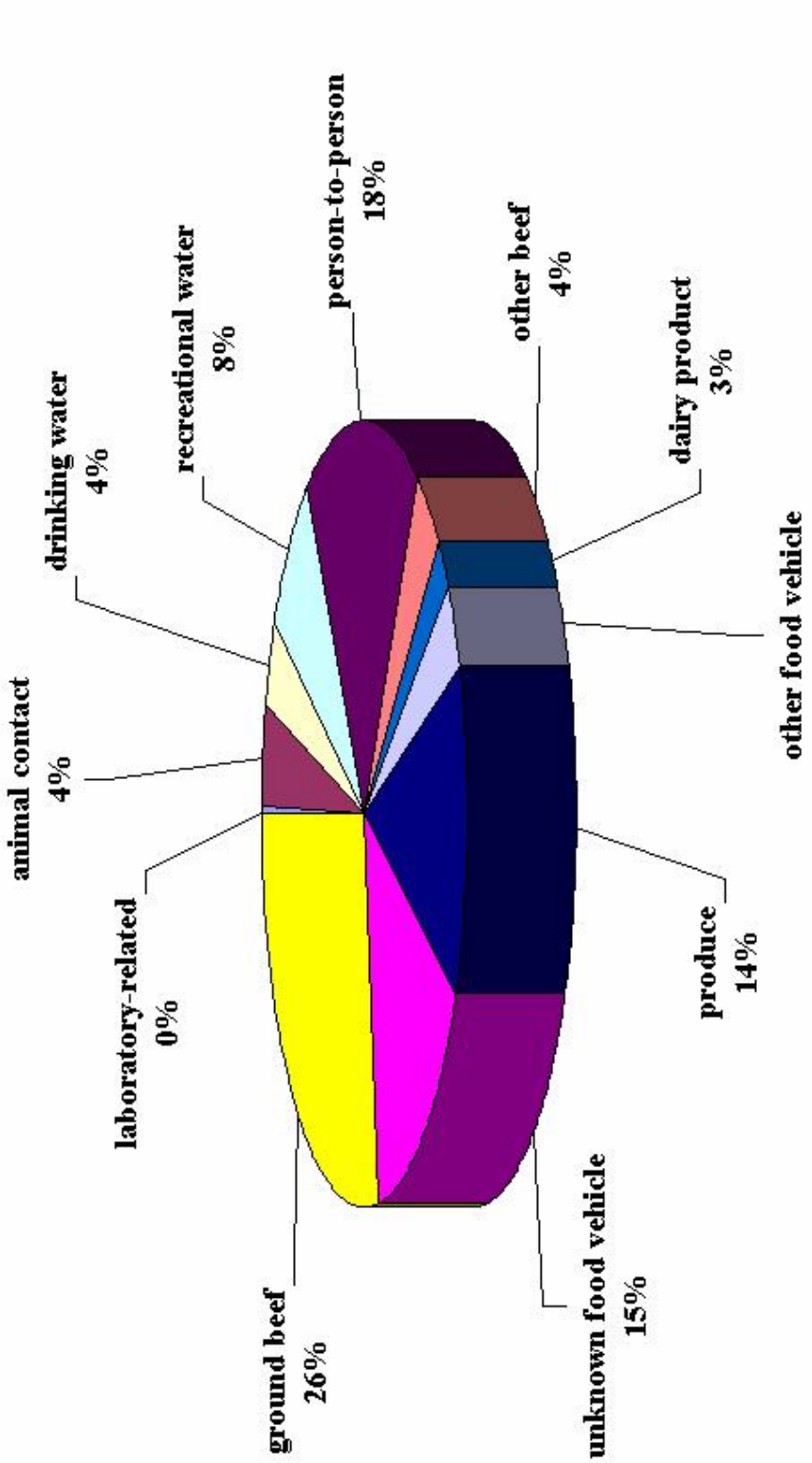

Outbreak number
Median size
Number of outbreaks
Median outbreak size
Year
0
5
10
15
20
25
30
35
40
45
50
60
1982
1984
1986
1988
1990
1992
1994
1996
1998
2000
2002

Figure 4. Transmission routes of EHEC O157:H7 infection (Rangel *et al.*, 2005a).

About 350 reported O157 outbreaks occurred between 1982 and 2002. Over 50% of the outbreaks originated from foodborne sources, with ground beef as the most frequent vehicle. Among the non-food related categories, person-to-person transmission contributed to the second highest incidence.


person-to-person
18%
other beef
4%
dairy product
3%
other food vehicle
produce
14%
unknown food vehicle
15%
ground beef
26%
laboratory-related
0%
animal contact
4%
drinking water
4%
recreational water
8%

(Rangel *et al*., 2005a). Due to the low-infectious dose of *E. coli* O157:H7 for humans, person-to-person transmission is a significant method of spread of the microbe, despite the fact that humans are incidental hosts. The fecal-oral route gives rise to outbreaks most frequently in child daycare centers (Galanis *et al*., 2003). Waterborne routes of transmission include recreational water, such as swimming pools and lakes, and contaminated drinking water; the latter source is an increasingly reported vehicle and has the potential to lead to much larger outbreaks (Hunter 2003; Craun *et al*., 2005). Other emerging modes of transmission include direct animal contact at petting zoos (Heuvelink *et al*., 2002) and laboratory-related incidents due to compromised safety procedures.

Natural reservoir of O157:H7

To understand the close correlation between *E. coli* O157:H7 and bovine-origin foods, one must take into the consideration that the natural reservoir of the organism is cattle. EHEC O157:H7 exists ubiquitously on a majority of cattle farms across the U.S. in both dairy and beef cattle. In 2002, the USDA's National Animal Health Monitoring System (NAHMS) conducted Dairy2002, NAHMS' third national study of U.S. Dairy operations. The study found that 38.5% of dairy farms had at least one cow that was culture positive. Infection with O157 is not associated with any recognizable disease in cattle; rather, the bacteria appear to be transient or "normal flora" *E. coli*. Juvenile cattle 3 to 18 months of age have a higher prevalence of O157 than either suckling calves or adult cattle (Cray, Jr. *et al*., 1995). The typical pattern of O157 shedding in a herd is one of epidemics of shedding interspersed with longer periods with no or only a few shedding

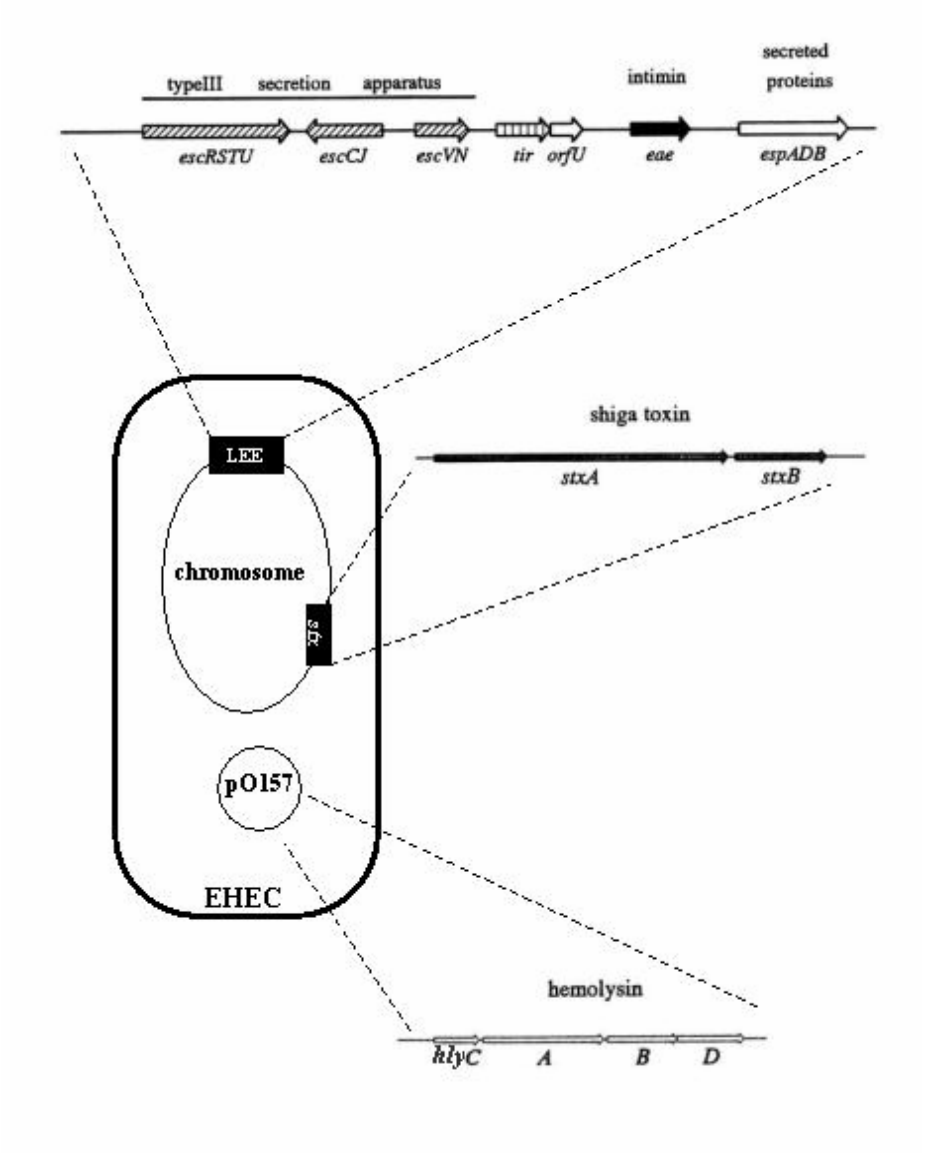
animals. The shedding epidemics occur mainly during warm weather, a period that coincides with peak summer outbreaks in humans (Chapman *et al*., 1997).

Pathogenic determinants

One paradigm in the study of steps in the pathogenesis of an infectious disease is exemplified in the following progression of events (Salyers *et al*., 1994). First, the pathogen gains entry into the host and evades the innate host defense. Next, the pathogen adheres to and colonizes the epithelial surface. Then, the pathogen replicates faster than the rate at which it is cleared, and during this time the agent may cause disease by excretion of exoproteins. Finally, the pathogen disseminates back into the environment via host shedding. The lifestyle of EHEC O157:H7 generally mirrors this pattern. A very low infectious dose of the organism is all that is needed to cause illness in humans: ingestion of fewer than 100 bacilli can result in disease (Tilden, Jr. *et al*., 1996). This low-dose model is in contrast to other enteric, bacteria such as *Salmonella* that typically requires 10^6-10^8 organisms to cause illness in the human host. One explanation for the low infectious dose of EHEC O157:H7 is that the glutamate-dependent acid-resistance system of the organism allows it to survive in the acidic environment of the stomach (Foster 2004). Additionally, the organism adheres closely to mucosal cells of the large bowel via a well-characterized adhesion molecule called intimin (Yu *et al*., 1992). Intimin is encoded in a pathogenicity island on the chromosome called locus of enterocyte effacement (LEE) (Fig. 5). In addition to intimin, LEE contains other factors in a type III secretion system that are critical for development of attaching-and-effacing (A/E) intestinal lesions that are characteristic of Enteropathogenic *Escherichia coli*

Figure 5. Virulence genes of EHEC O157:H7.

The locus of enterocyte effacement (LEE) is a pathogenicity island located on the chromosome. There are more than 40 different genes, organized into three major regions (Frankel *et al*., 1998), encoded on the LEE that are essential for attaching and effacing (A/E) lesions typical of many EHEC and enteropathogenic *E*. *coli* (EPEC) strains. The first region contains genes for a type III secretion system; the second region encodes intimin and the translocated intimin receptor (Tir); and, the third region is comprised of loci for several secreted effector proteins (Esp) that are important in the modification of host cell signal transduction during the formation of A/E lesions. The *stx* operons are encoded on lysogenic phages on the chromosome, with the gene for the A subunit immediately upstream of the gene for the B subunit. Several different types of *stx*-phages, some of which are defective, may be present simultaneously on the chromosome of a single EHEC strain (Jackson *et al*., 1987; Schmitt *et al*., 1991). The large virulence plasmid of O157:H7, pO157, encodes an EHEC hemolysin (Schmidt *et al*., 1995) and a type II secretion system (Schmidt *et al*., 1997).


typeIII secretion apparatus
intimin
secreted proteins
escRSTU
escCJ
escVN
tir
orfU
eae
espADB
shiga toxin
stxA
stxB
LEE
chromosome
stx
pO157
EHEC
hemolysin
hlyC
A
B
D

(EPEC) and EHEC infections. EHEC O157:H7 also harbors a large virulence plasmid designated pO157 (Toth *et al*., 1990) (Fig. 5). The 90-kb plasmid encodes a hemolysin called enterohemolysin that has been proposed to facilitate the acquisition of iron by the organism from the blood released in the intestine (Law *et al*., 1995). Lastly, and perhaps the most important virulence factor of EHEC O157:H7 is Shiga toxin (Stx), a cytotoxin that is critical to the development of hemorrhagic colitis and the onset of HUS.

Diagnosis

The diagnosis of EHEC O157:H7 infection should be considered for any previously healthy person who reports visible blood in their stools and other symptoms. Various differential and selective media are available for screening O157:H7 strains on the basis of their biochemical characteristics. Unlike most other *E. coli* serotypes, O157:H7 does not ferment sorbital and thus forms colorless colonies on sorbital-containing MacConkey (SMAC) agar (March *et al*., 1986). Culturing stool samples on SMAC agar provides a simple, economical, and overall, reliable diagnostic screen for *E. coli* O157:H7 strains. Cefixime and tellurite can be added to SMAC agar (CT-SMAC agar) to improve selectivity and differentiation by permitting the growth of Stx-producing *E. coli* O157:H7 (and *Shigella sonnei* strains) while inhibiting the growth of most of the other *E. coli* isolates (Zadik *et al*., 1993). Another feature of *E. coli* O157:H7 that distinguishes it from most other *E. coli* serotypes is its inability to produce β-$_{D}$-glucuronidase whose substrate is 4-methyl-umbelliferyl-$_{D}$-glucuronide (MUG) (Thompson *et al*., 1990). Non-O157 *E. coli* can hydrolyze MUG to produce a fluorescent compound that yields a spectrum of colored colonies when grown on MUG-containing media; by constrast, O157

colonies are colorless on that media. The low dose of *E. coli* O157:H7 that can lead to disease also often results in extremely low numbers of the organism in stool samples. Therefore, an enrichment step that involves immunomagnetic separation with magnetic beads coated with anti-O157 antibody is sometimes used before culturing samples on differential media (Wright *et al*., 1994). Suspected colonies from various screening methods need to be confirmed as *E. coli* O157:H7 by immunological assays (such as slide agglutination reactions) with appropriate antibodies. Most immunoassays designed to detect either the O157 LPS and H7 flagellar antigens, or antibodies to them, or Stx are commercially available in ELISA-based kits(Law *et al*., 1994; Padhye *et al*., 1991; Kim *et al*., 1992). DNA probes and PCR techniques have also been developed to detect presence of virulence genes such as *stx* and *eae* on the chromosome and *hly* on pO157. Lastly, testing for Stx activity in stool samples has been shown to be a sensitive approach for the detection of Stx-producing organisms when conventional culture techniques have failed to yield the agent (Karmali 1989).

Treatment

Treatment of patients with EHEC infection is primarily limited to supportive care. A confounding problem is that antibiotic therapy in these individuals is not recommended because some of the antimicrobial agents commonly used for gastrointestinal infections can cause the bacteria to lyse (are bactericidal) and release the toxin (Walterspiel *et al*., 1992). Additionally, certain antibiotics can induce the lytic cycle of Stx-phages and thus cause the bacteria to produce more toxins (Acheson *et al*., 1989). Indeed, a cohort study with children infected with *E. coli* O157:H7, Wong and colleagues concluded that

antibiotic treatment actually increased the risk of the hemolytic-uremic syndrome (Wong *et al*., 2000). However, one study conducted during the 1996 outbreak in Japan showed that administration of fosfomycin reduced the risk of HUS (Takeda 1998). Furthermore, a recent meta-analysis that reviewed all published epidemiological studies of O157:H7 and HUS from 1983 to 2001 showed no higher risk of HUS associated with antibiotic administration (Safdar *et al*., 2002).

The controversy over antibiotic usage remains to be resolved. On the other hand, the usage of anti-motility agents is universally contradicted for the treatment of EHEC infection because slowing intestinal movement could aid in the colonization of the organism and thus decrease clearance of the bacteria and the toxin. Treatments for HUS sometimes involves renal dialysis and /or plasmapheresis but generally always include management of fluid and electrolyte balance and nutritional support. Other therapeutic agents, such as receptor mimics that bind Stx are currently under development (Takeda *et al*., 1998; Armstrong *et al*., 1995; Mulvey *et al*., 2003). One of these compounds is Synsorb-Pk, an oral drug comprised of a synthetic analog of globotriaosylceramide (Gb_3), the eukaryotic receptor for Stx, designed to absorb Stx from the intestines of patients with blood diarrhea in the hopes of preventing HUS (Armstrong *et al*., 1995). However, the phase III randomized study of Synsorb-Pk failed to prove that oral therapy with this toxin-binding agent can diminish the severity of disease in children with *E. coli*-associated HUS (Trachtman *et al*., 2003). The same group of researchers who designed Synsorb-Pk has also developed a newer version, called Daisy (Mulvey *et al*., 2003). Promising *in vivo* data showed that Daisy protected mice against both Stx1 and Stx2 challenge (Mulvey *et al*., 2003).

Shiga Toxin

History and nomenclature

In 1977, Konowalchuk *et al*. observed that a toxin found in culture filtrates of a number of *E*. *coli* strains isolated from patients with bloody diarrhea had irreversible cytopathic effects on African green monkey (Vero) cells (Konowalchuk *et al*., 1977); these investigators then coined the term VT (for toxic on Vero cells) to describe this cytotoxin. In 1983, the same year that Riley *et al*. reported the first isolation of O157:H7 from patients with hemorrhagic colitis (HC), Karmali *et al*. noted that filtrates of stools from patients with the hemolytic uremic syndrome who had had a preceding bout of HC were positive for a cytotoxin active on Vero cells (Karmali *et al*., 1983). These researchers denoted the fecal cytotoxin (VT) for Vero toxin (Karmali *et al*., 1983). Concurrently, O'Brien *et al*. purified and characterized the cytotoxin produced by one of Konowalchuk's *E*. *coli* strains (H30) and noted the similarities between this cytotoxin and the Shiga toxin (Stx) produced by *Shigella dysenteriae* type 1, both in structure and biological activity; thus, the term Shiga-like toxin (SLT) was created (O'Brien *et al*., 1983a). In addition, O'Brien and colleagues observed that the cytotoxic effects of SLT on HeLa cells was neutralizable with antiserum to Stx (O'Brien *et al*., 1982) and subsequently showed that SLT and VT were the same toxin and were produced by the O157:H7 strains described by Riley *et al*. (O'Brien *et al*., 1983b).

The system of nomenclature of O157:H7 then became confusing: some groups referred to the cytotoxin as SLT because of the similar properties it shared with Shiga toxin produced by *S*. *dysenteriae* type 1, while other groups used the name VT for the

toxin's cytotoxic effect on Vero cells. To make matters worse, two types of SLT/VT were later described: SLT-I or VT1, and SLT-II or VT2. In addition, more subtypes of SLT-II or VT2 were also identified, e.g. SLT-IIc or VT2c. In an attempt to clear up the confusion, a new nomenclature system was proposed wherein the gene and the protein designations for the related toxins were renamed after the prototype Stx that was originally described by the Japanese scientist Dr. Shiga over a hundred years ago (Calderwood *et al*., 1996). The nomenclature proposed by Calderwood and others is used hereafter in this dissertation and is shown in Table 1.

Structure and mode of action

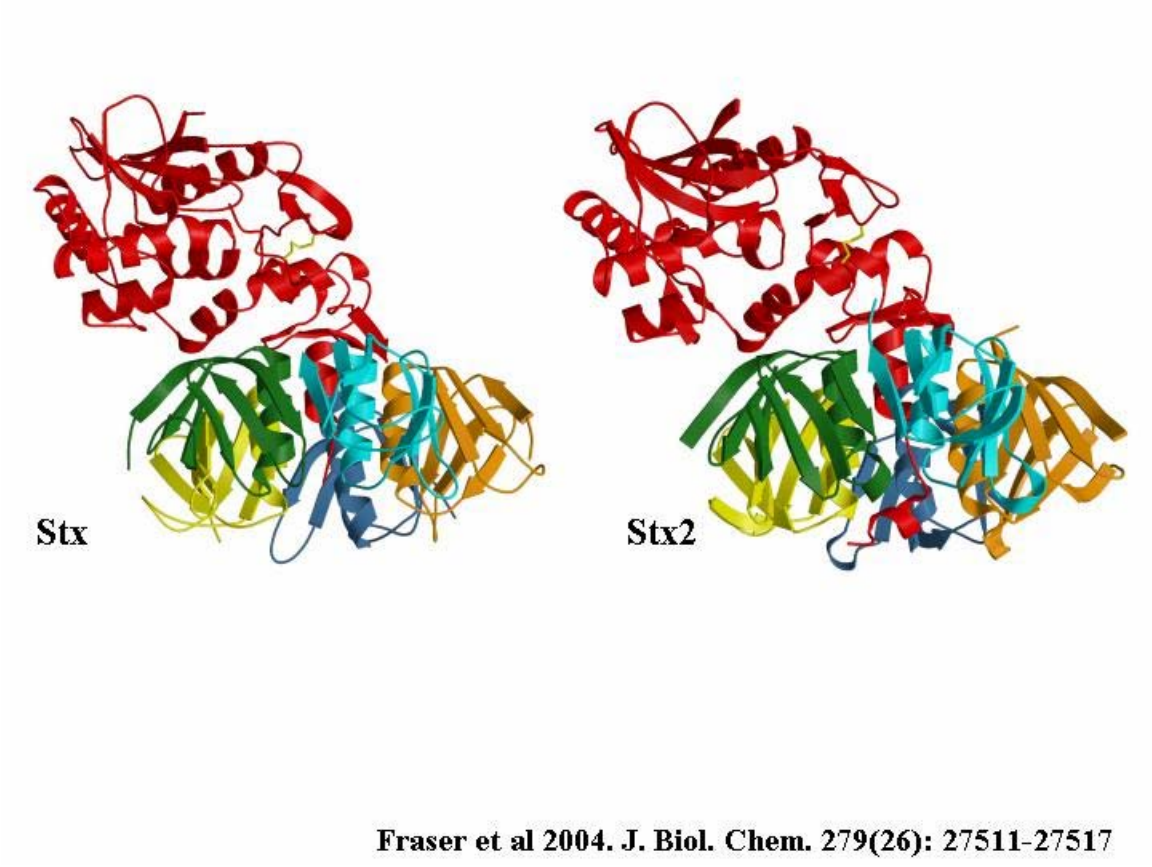
Shiga toxin (Stx) types 1 and 2 are also referred to as serotypes because neutralizing antiserum to the homologous type does not block the activity of the heterologous type (Strockbine *et al*., 1986). However, Stx1 and Stx2 share similar AB_5 holotoxin structures as well as modes of action (Fig. 6). The B subunits (~7 kDa per monomer) form a pentameric ring that is responsible for binding to the eukaryotic cellular receptor, Gb_3 (Jacewicz *et al*., 1986). The A subunit (~ 32kDa) can be cleaved by trypsin to yield A_1 (~28 kDa) and A_2 (~4kDa) polypeptides. The A_1 portion is the enzymatic domain of the toxin, and the glutamate residue at position 167 is critical for this *N*-glycosidase activity (Endo *et al*., 1988; Gordon *et al*., 1992a). The A_2 peptide inserts through the B pentameric ring and is thought to be responsible for noncovalently linking the A_1 portion to the B pentamer and, thus, for holotoxin assembly (Austin *et al*., 1994). To intoxicate a cell, each monomer in the pentamer binds up to three molecules of Gb_3 at distinct binding

Table 1. Nomenclature of members of the Shiga toxin family

Previous names	Proposed new nomenclature	
	Gene	Protein
Shiga toxin (Stx)	*stx*	Stx
Shiga-like toxin I (SLT-I) or verotoxin 1 (VT1)	stx_1	Stx1
SLT-II or VT2	stx_2	Stx2
SLT-IId or VT2d	stx_{2d}	Stx2d

Figure 6. The crystal structures of Stx and Stx2 (Fraser *et al.*, 2004).

Stx1 and the prototype Stx from *Shigella dysenteriae* type 1 are essentially identical with one amino acid difference, whereas Stx2 shares only 56% amino acid homology with Stx/Stx1. The A-subunit is red; the B-subunits are yellow, green, blue, cyan, and orange.



Fraser et al 2004. J. Biol. Chem. 279(26): 27511-27517

sites (Fraser *et al*., 2004) and the holotoxin is then endocytosed through the clathrin-coated pits (Sandvig *et al*., 2000). Then, via the retrograde transport pathway, the toxin is transported first to the Golgi apparatus then to the endoplasmic reticulum. The A subunit is then translocated to the cytoplasm, where it acts on the 60S ribosomal subunit to remove a critical adenine residue from the 28S rRNA (Endo *et al*., 1988). This cleavage of a purine from 28S rRNA by Stx results in inhibition of protein synthesis and cell death.

Pathogenesis of Stx-mediated HUS

The connection between Stx and HUS was first recognized by Karmali *et al*. (Karmali *et al*., 1983; Karmali *et al*., 1985). This link between toxin and disease was further supported by the association of Stx-producing *Citrobacter freundii* isolates with HUS cases in a nursery school (Tschape *et al*., 1995). Upon ingestion of Stx-producing *E. coli*, Stx is first released from the bacteria that are adhered to the intestinal epithelium; then the toxin crosses the intestinal barrier and enters the systemic circulation; lastly Stx reaches the kidney and other target organs (Fig. 7). The exact mechanism of how Stx is translocated from the gut to the systemic circulation has yet to be delineated. Acheson *et al*. demonstrated *in vitro* that Stx1 can be translocated from the apical to basolateral surface of human intestinal cells without apparent cellular damage (Acheson *et al*., 1996b). Since one of the cell lines in that study, T84, does not express Gb3 (Ismaili *et al*., 1995), the translocation of the toxin observed is most likely to be via a receptor-independent route. Schuller and collegues (Schuller *et al*., 2004) confirmed the findings of Acheson *et al*. and showed that Gb3-negative intestinal epithelial cells are able to

endocytose Stx1 but remain resistant to cytotoxicity; however, in the same study, these investigators demonstrated that Stx2 can induce cellular damage to intestinal epithelium via a receptor-independent mechanism. Once Stx translocates from the gut epithelium into the circulation, the toxin may be transported through the blood by sticking to certain cells. Indeed, *in vitro* studies have shown that Stx can bind to human B lymphocytes (Cohen *et al*., 1990), the P blood group antigen of human erythrocytes (Bitzan *et al*., 1994), monocytes (Cohen *et al*., 1990; Bitzan *et al*., 1994), and/or polymorphonuclear leukocytes (te Loo *et al*., 2000). That Stx is not in a cell-free state in the circulation is suggested by the fact that detection of Stx in the blood of an HUS patient has only been reported once by te Loo *et al*. (Te Loo *et al*., 2001). Nevertheless, the toxin is clearly made *in vivo* at sufficient levels to be antigenic because antibodies to Stx have repeatedly been found in the sera of HUS patients (Lopez *et al*., 1991; Greatorex *et al*., 1994; Ludwig *et al*., 2002b). In spite of the lack of direct evidence (with the exception of the data of Te Loo and colleagues cited above), the general consensus in the field is that Stx is transported through the circulation to its ultimate target, the Gb3-expressing tissues in the glomeruli of the kidney.

The pathological mechanism that underlies the development of HUS is not fully understood but may reflect the direct cytotoxic action of Stx on glomerular endothelial cells that express high levels of Gb3. Such glomerular cell damage could lead to the observed constriction of capillary lumina and occlusion of glomerular microvasculature with platelets and fibrin (Louise *et al*., 1995). This occlusion, in turn, may result in a decreased glomerular filtration rate or renal failure and erythrocytes damage, features that are typical of HUS.

The immune response during an EHEC infection may also play a role in the development of Stx-mediated HUS. Studies have shown that proinflammatory cytokines such as tumor necrosis factor (TNF)-α or interleukin (IL)-1β induced by bacterial lipopolysaccharide (LPS) can upregulate Gb3 expression on cultured human endothelial cells, and, thus, increase susceptibility of the cells to Stx-induced injury (van de Kar *et al*., 1992; Van Setten *et al*., 1997). Karpman *et al*. and van de Kar *et al*. also reported increased levels of IL-6 in the sera and urine of HUS patients, and these IL-6 levels correlated with the severity of the observed diseases (Karpman *et al*., 1995; van de Kar *et al*., 1995).

The type of Stx produced by the infecting EHEC strain also influences the development of HUS. Epidemiological studies have shown that infections with EHEC strains that produce Stx2 alone are more likely to progress to HUS than are those infections with strains that produce Stx1 alone or, both Stx1 and Stx2 (Ostroff *et al*., 1989; Pickering *et al*., 1994). Although the two toxin types are related by structure and function, they vary in several ways that may contribute to the stronger the link between expression by an EHEC O157:H7 strain of Stx2 versus Stx1 and HUS. These differences between Stx1 and Stx2 are as follows. First, the production of Stx1 is at least partially iron regulated *in vitro* (Sung *et al*., 1990), whereas the production of Stx2 is completely controlled by phage induction (Wagner *et al*., 2001). Second, the binding affinity between Stx1 and Gb_3 is 10 times higher than between Stx2 and Gb_3 (Head *et al*., 1991). Our laboratory has speculated that this increased affinity of Stx1 for its receptor makes it more likely than Stx2 to be irreversibly bound in the Gb_3-filled gut (unpublished data from mice) and not adsorbed systemically. Third, Stx2 is more heat- and pH-stable than

Stx1 (Tesh *et al*., 1993). Fourth, Stx2 has a 50% lethal dose (LD_{50}) that is 400 times lower than that of Stx1 when administered in a purified form intraperitoneally or intravenously to mice (Tesh *et al*., 1993). Fifth, the renal damage observed in the streptomycin-treated mice is due to Stx2 when the mice are orally infected with an EHEC strain that produces both Stx1 and Stx2 (Wadolkowski *et al*., 1990). In that study, passive administration of monoclonal anti-Stx2 but not anti-Stx1 prevented the tubular necrosis and death in mice. Taken together, the net effect of these differences between Stx1 and Stx2 may form the basis of the stronger association between Stx2 and HUS, though EHEC strains that produce only Stx1 are still capable of causing HUS (Jelacic *et al*., 2002).

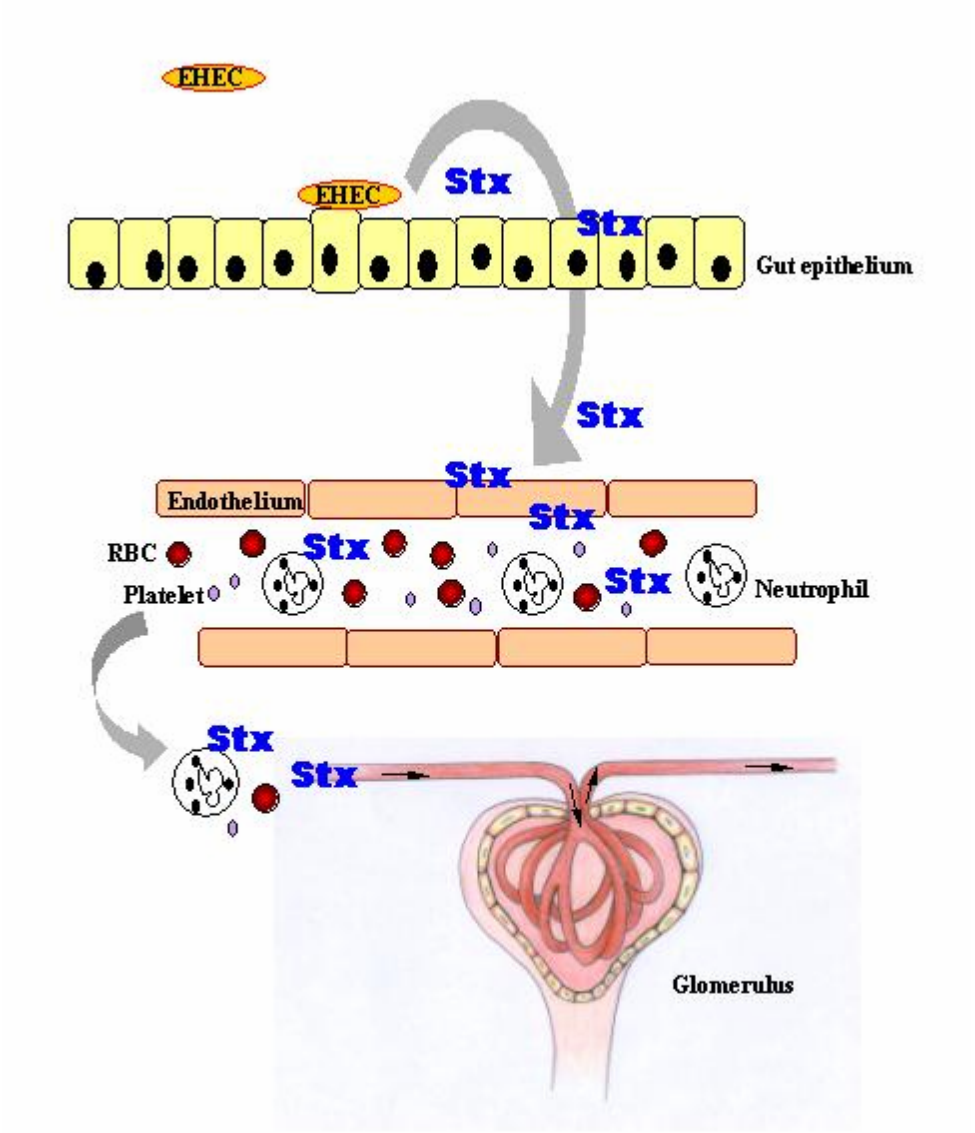
Vaccine development to protect against Stx-mediated illness

Presently, there is no FDA-approved vaccine for Stx alone or for EHEC O157:H7 infection. Both *in vivo* and *in vitro* data show that toxin-neutralizing antibodies are effective at preventing intoxication by Stx, whether by blocking receptor binding (Strockbine *et al*., 1985) or by impeding the catalytic action (Perera *et al*., 1988) of the toxin. One well-established approach to induce neutralizing anti-toxin antibodies is to use toxoids, derived from chemical inactivation or genetic mutation, as vaccines. Chemical toxoiding of Stxs has raised issues such as alteration of conformation (Metz *et al*., 2003) and residual toxicity (Gordon *et al*., 1992b). Genetically created toxoiding is a more appealing approach than chemical inactivation but issues such as spontaneous reversion of the toxoid in a live expression strain still need to be addressed. Other efforts have focused on subunit vaccines, especially the B-subunit of Stx (Boyd *et al*., 1991;

Acheson *et al*., 1996a; Marcato *et al*., 2001). Additionally, a DNA vaccine has also been reported for Stx2 (Capozzo *et al*., 2003). We hypothesize that a multivalent vaccine that contains protective epitopes from both types of Stxs would be most likely to offer the broadest protection against the development of the life-threatening HUS in individuals infected with O157 or other Stx-producing *E.coli* (STEC).

Figure 7. Overview of a proposed pathway by which Stx is trafficked from the gastrointestinal tract to the kidney.

The Stx-producing bacteria enter the gut and adhere to the intestinal epithelial cells. *In vitro* evidence suggests that Stx released from the bacterium is then translocated from the apical to the basolateral side of the epithelium (Acheson *et al*., 1996b). Stx is then released into the submucosa and targets Gb_3-expressing endothelial cells of the intestinal capillary network, an interaction that may result in hemorrhagic colitis (Jacewicz *et al*., 1999). The injured endothelial cells then release inflammatory cytokines, such as IL-8, that are important in the recruitment of neutrophils to the lamina propria of the intestine. Such an increase in inflammatory cytokines have been observed in patients with HC-associated HUS (Fitzpatrick *et al*., 1992). Stx subsequently enters the systemic circulation to reach the kidney and damage glomerular endothelial cells, a critical pathological event in the pathogenesis of HUS. *In vitro* data suggests that Stx can bind to neutrophils (te Loo *et al*., 2000) and monocytes (Cohen *et al*., 1990; Bitzan *et al*., 1994) and induce cytokine release (Van Setten *et al*., 1996), which, in turn, upregulates the expression of Gb_3 and sensitize glomerular cells to the Stx (Ray *et al*., 2001).


EHEC
EHEC
Stx
Stx
Gut epithelium
Stx
Stx
Endothelium
Stx
RBC
Stx
Platelet
Stx
Neutrophil
Stx
Stx
Glomerulus

Intimin

History

Intimin is an outer membrane protein (OMP) found not only in EHEC, but also in EPEC, *Citrobacter rodentium*, and *Escherichia albertii* (formerly known as *eae*-positive *Hafnia alvei*). The main function of this OMP is to meditate intimate adherence of the bacterium to epithelial cells (hence the name intimin) as part of a multi-step series of bacterial- host cell interactions that results in a unique attaching-and-effacing (A/E) histopathology (Donnenberg *et al*., 1992) (Fig 8). In 1969, Staley *et al*. reported that a strain of *E*. *coli* serotype O55:H7 colonized the intestines of newborn pigs and that the brush border microvilli of the epithelial cells were destroyed, or effaced, at the site where the bacteria were intimately attached (Staley *et al*., 1969). In 1983, Moon *et al*., used a O55:H7 that was later recognized as an EPEC strain to confirm the association between EPEC virulence and the presence of these attaching and effacing lesions which he demonstrated in the intestines of infected gnotobiotic piglets and in inoculated ligated intestinal loops of pigs and rabbits (Moon *et al*., 1983). Knutton *et al*. (Knutton *et al*., 1989) discovered that the pedestal formation, characterized by Moon *et al*., underneath the intimately attached bacteria was a result of a high concentration of polymerized filamentous actins (F-actin). This key observation led to the development of the fluoresent-actin staining (FAS) test, in which the fluorescein isothiocyanate (FITC)-labled phalloidin binds specifically to the polymerized F-actins in infected cells.

The FAS test is not only useful for categorization of strains EPEC or EHEC strains but is also an effective screening method for identification of bacterial genes involved in the A/E formation. By using the FAS test to screen for Tn*pho*A mutants of EPEC that

have A/E-negative phenotype, Jerse *et al*. (Jerse *et al*., 1990) discovered the gene encoding intimin, (*eae*, for *E. coli* attaching and effacing). Subsequently, Yu and colleagues identified a homologous copy of *eae* in an EHEC O157:H7 strain that shares 83% similarity in deduced amino acid sequence with EPEC intimin (Yu *et al*., 1992).

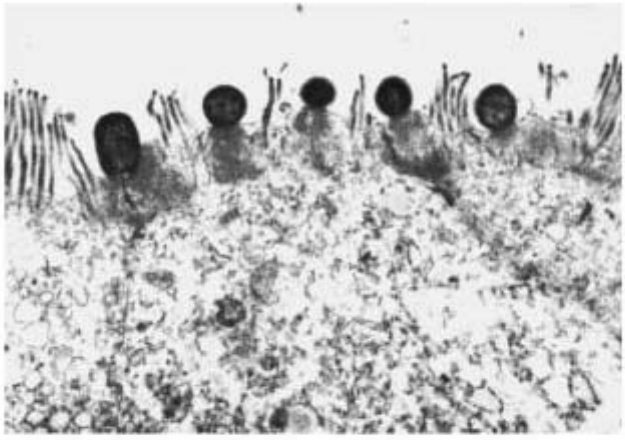
Role of intimin in A/E adherence

All genes responsible for A/E formation in EPEC and EHEC are encoded on a pathogenicity island called Locus for Enterocyte Effacement (LEE) (McDaniel *et al*., 1995; McDaniel *et al*., 1997). These include loci that encode a type III secretion system (TTSS), effector proteins secreted through the TTSS, as well as intimin and the translocated intimin receptor (Tir) (Fig. 8). The phenotypes and the process of EPEC- and EHEC-induced A/E formations are very similar with a few exceptions. First, the bundle forming pili (BFP) of EPEC mediate the initial adherence of bacteria to the intestinal epithelium; EHEC lacks the BFP. Following the initial attachment of EPEC or EHEC to epithelial cells, the bacteria secrete several effector proteins into the host cell via the TTSS that trigger a number of signal transduction pathways. As a consequence of these signaling events, phospholipase C (PLC) and protein kinase C (PKC) are activated, fluxes occur in the intracellular concentration of inositol triphosphate (IP_3), and Ca^{2+} is released from intracellular storage into the cytosol (Wolff *et al*., 1998). The increased intracellular concentration of Ca^{2+} is critical for the polymerization of actin and subsequent A/E lesion formation (Baldwin *et al*., 1992). The EPEC or EHEC then closely adhere to the cell by a mechanism that involves secretion of the bacterially

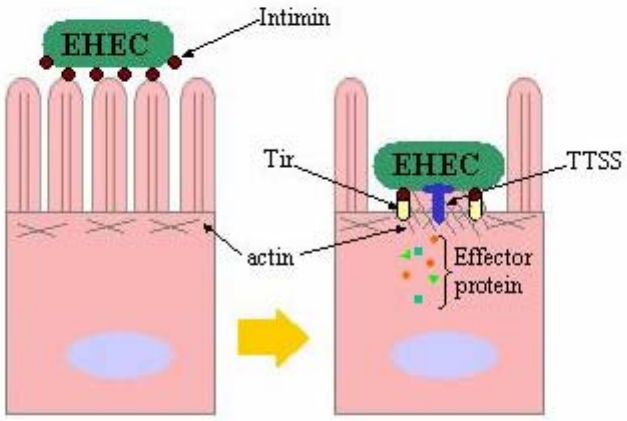
Figure 8. Attaching and effacing lesion.

A: electron micrograph of the EHEC closely attached to the luminal side of an enterocyte membrane. In some instances, a pedestal with electron dense material (actin) is evident directly below the bacterium. Localized disruption of the brush border microvilli is also evident (http://www.sat.affrc.go.jp/joseki/O157/O157_ae02.jpg). **B:** schematic diagram illustrating stepwise A/E formation. First, initial extracellular surface adherence is mediated by intimin binding to host surface proteins such as nucleolin (Sinclair *et al*., 2002). Then, through the type III secretion pathway, various bacterial effector proteins including Tir are translocated into the host cell, which result in intimin-Tir binding, microvilli effacement, and actin polymerization.

A



B



encoded receptor for intimin, Tir, into the host-cell membrane. Tir then binds to intimin on the outer membrane of the bacteria (Rosenshine *et al*., 1996). The net effects of these actions result in cytoskeletal rearrangements and pedestal formation underneath the intimately attached bacterium as well as the localized effacement of the brush border microvilli at the site of the bacterial adherence. The differences between EPEC and EHEC intimate attachment are: 1) after translocation into host cell, EPEC Tir needs to be phosphorylated on one or more tyrosine residues to function as the receptor for intimin while tyrosine phosphorylation of EHEC Tir is not essential for intimin binding (Kenny *et al*., 1997); 2) different types of intimin contribute to tissue tropisms observed in EPEC- and EHEC- infections(Tzipori *et al*., 1995a; Phillips *et al*., 2000) . In addition to Tir, other host cell intimin receptors have been identified as beta-1 (β1) integrins for EPEC and EHEC intimins (Frankel *et al*., 1996) and nucleolin for EHEC, EPEC, and *C. rodentium* intimin (Sinclair *et al*., 2002).

Intimin as a vaccine candidate

Levine *et al*. (Levine *et al*., 1985) reported that volunteers who ingested a virulent strain of EPEC mounted serum IgA and IgG responses to a 94-kDa plasmid-associated outer membrane protein that was later identified as intimin. The study also showed that serum antibodies to intimin were protective against the oral EPEC challenge. In another human challenge study with the same strain of EPEC and its isogenic *eae* null mutant, the number of diarrhea cases was significantly lower in the group that ingested the *eae* mutant (Donnenberg *et al*., 1993a). The data from these volunteer experiments suggest that intimin is a good vaccine candidate to protect against EPEC disease. EHEC, on the

other hand, can produce a much more severe disease outcome than EPEC; therefore the study of the involvement of intimin in EHEC disease is limited to animal models and *in vitro* systems.

Compelling evidence has accumulated to demonstrate the pivotal role intimin plays in the adherence of EHEC to epithelial cells. In gnotobiotic piglet model, intimin is absolutely essential for EHEC colonization and A/E lesion formation in the large intestines (Donnenberg *et al*., 1993b; McKee *et al*., 1995; Tzipori *et al*., 1995b). In addition, Mckee and O'Brien demonstrated that antibodies directed against C-terminal third of EHEC intimin can block adherence of O157:H7 to HEp-2 cells (McKee *et al*., 1996). The presence of intimin-specific antibodies in the sera of HUS patient indicates that intimin is at least produced during human infections with EHEC and may suggest a role for intimin in disease; however, bloody diarrhea and HUS are also observed in patients infected with *eae*-negative strains of Stx-producing *E. coli*. Furthermore, given the extremely low infectious dose of EHEC and extreme potency of Stxs, the use of EHEC intimin alone as a vaccine candidate to reduce colonization of humans is a less than ideal approach. However, we do propose intimin alone could serve as a vaccine candidate in ruminant animals such as cattle, the natural reservoir of EHEC O157:H7 and the main source of transmission to human. Our reasoning is that studies have shown that EHEC intimin is required for the colonization of O157:H7 in neonatal calves and adult sheep and cattle (an-Nystrom et al., 1998; Cornick et al., 2002) and that the animals do not need to be protected from the systemic action of Stxs delivered from the gut .

Transgenic Plant-based Vaccines

Unique advantages of plant vaccines

Transgenic plants have been engineered to produce recombinant proteins for a variety of reasons. One such application is in vaccine development whereby transgenic plants that express antigens from various pathogens are given to animals or human as a means of immunization. Plant-based vaccines offer many advantages over conventional vaccine development strategies. Foremost, the vaccine can be delivered by ingestion of the edible part of the transgenic plant. The plant cell wall acts as a capsule that protects the antigens from digestive degradation (Streatfield 2005). Oral administration is not only safer than needle and syringes but also reduces the need for trained medical personnel. Unlike conventional tissue culture, plant-derived vaccines have a very low risk of contamination with animal pathogens (Cimolai 2001). In addition to the safety reason, the costs of plant vaccines are significantly lower than those manufactured by conventional methods in the areas of production, packaging, storage, and transportation. The transgenic plants can be easily expanded for high-yield field production (Streatfield *et al*., 2003). Freeze-drying of plant tissue, as a way of food processing, can be applied to both concentrate the antigen and stabilize it in a dried form that can be stored at room temperature(Tacket 2004). This process not only lowers the cost of packaging and shipping but also provides a practical vaccine strategy for the developing world where maintenance of a cold-chain for vaccine stability is not generally feasible.

Examples of plant vaccines

There are a number of plant-derived vaccines developed against diarrheal diseases. The B subunit of heat-labile enterotoxin (LT-B) of enterotoxigenic *E. coli* (ETEC) was expressed in both transgenic potatoes (Haq *et al*., 1995) and corn (Streatfield *et al*., 2001), and formed multimers conformationally similar to those generated in a bacterial expression system. In the subsequent clinical studies, volunteers who ingested the transgenic plant material developed LT-B specific IgG and IgA (Tacket *et al*., 1998; Tacket *et al*., 2004). Similarly, the B subunit of cholera toxin (CT-B) was expressed in potatoes and induced both serum and mucosal anti-CT-B antibodies in mice that were fed the transgenic potatoes (Arakawa *et al*., 1997). Moreover, Judge *et al*. reported that transgenic tobacco cells that expressed intimin reduced the colonization of EHEC O157:H7 in mice that were fed the plant material (Judge *et al*., 2004). Mason *et al*. expressed the capsid protein of Norwalk virus in tobacco and potatoes. When the transgenic potatoes were fed to mice, serum and fecal antibody responses were detected (Mason *et al*., 1996). More recent development of transgenic plant vaccines include those against foot-and-mouth disease, tuberculosis, measles, and SARS (Huang *et al*., 2005; Rigano *et al*., 2006; Webster *et al*., 2005; Pogrebnyak *et al*., 2005). In aggegrate, these results show that transgenic plants are efficient vectors for expression of bacterial and viral antigens and that plant-derived antigens resemble the native antigen as indicated by their capacity to induce specific, protective immune responses upon oral immunization.

Specific Aims of This Dissertation

The goal of this dissertation is to further the advancement of vaccine development against EHEC O157:H7. The central hypothesis of this work is that plant-derived vaccines can be used to both eliminate Stx-mediated illness and reduce the transmission of EHEC O157:H7 from animal reservoirs to humans. The specific aims of this dissertation are as follows:

1. Generate genetically engineered toxoids of Stx1 and Stx2 as vaccine candidates and evaluate them in a dual-toxin lethal challenge mouse model to show that genetic toxoids are immunogenic and protective and to confirm or refute the notion that a multivalent toxoid vaccine is required to protect against strains of O157:H7 that produce both types of the Stx.
2. Develop a plant-based vaccine to protect against Stx2 intoxication in an oral infection challenge in mouse model by: first, engineering transgenic tobacco cell lines to express Stx2 toxoid; then, characterizing the plant-derived toxoid; and lastly, feeding the transgenic tobacco cells to mice as a means of oral immunization to protect against subsequence intoxication challenge.
3. Continue an effort to express EHEC-intimin in transgenic maize as an oral vaccine to impede the colonization of O157:H7 in cattle and other reservoir animal species.

Chapter Two

Genetic toxoids of Shiga toxin types 1 and 2 protect mice against homologous but not heterologous toxin challenge

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Note: all of the figures and tables shown reflect the work of Sharon Wen with the exception of the Stx2 toxoid construct that was created by Dr. Nicole Judge. Drs. Teel and O'Brien contributed to the design of the experiments and interpretation of the data as well as the preparation of the manuscript.

Abstract

Shiga toxin type 1 (Stx1) and type 2 (Stx2) are produced by *Escherichia coli* O157:H7 and are responsible for the life-threatening sequela, the hemolytic uremic syndrome. Whether antisera to Stx1 or Stx2 are cross-neutralizing remains controversial, so we constructed genetic toxoids of Stx1 and Stx2 and evaluated them as vaccines. Antisera from mice immunized with a single toxoid type recognized and neutralized the homologous toxin but not the heterologous toxin. Furthermore, only mice immunized with Stx1 and Stx2 toxoids were protected against a lethal challenge of both toxins. We conclude that Stx1 and Stx2 are distinct antigens for mice.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 cause an estimated 73,000 illnesses in the United States annually (Rangel *et al*., 2005b). Infection is associated with diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS), a life-threatening sequela characterized by hemolytic anemia, thrombocytopenia, and renal failure. HUS is mediated by Shiga toxin (Stx) produced by strains that harbor toxin-converting phages (Karmali *et al*., 1983; Newland *et al*., 1985; O'Brien *et al*., 1989).

The two main serotypes of Shiga toxins produced by EHEC are Stx1 and Stx2. Stx1 is virtually identical to the prototype Shiga toxin from *Shigella dysenteriae* type 1. Two related variants of Stx1, Stx1c and Stx1d, have been described in *E. coli* strains of human and animal origin, respectively (Zhang *et al*., 2002; Burk *et al*., 2003). There are several variants of Stx2 (Stx2c, Stx2d-activatable, Stx2e, Stx2f) that are antigenically related to Stx2 but exhibit somewhat different biological activities (Melton-Celsa *et al*., 1996; Lindgren *et al*., 1994; Perera *et al*., 1988; Samuel *et al*., 1990; Schmidt *et al*., 2000). *E. coli* strains that produce one or both types of Shiga toxins and Stx2 variants are common (Feder *et al*., 2003; Schmitt *et al*., 1991; Strockbine *et al*., 1986). All of the Shiga type toxins have the same 1A:5B holotoxin structure and N-glycosidase activity, but Stx1 and Stx2 share only 55% homology by amino acid sequence (Kaper *et al*., 1998a).

Reports in the literature about the degree of antigenic cross-reactivity between the Stx/Stx1 and the Stx2 groups are contradictory. On the one hand, Donohue-Rolfe, *et al*. reported that a monoclonal antibody against the Stx2B subunit recognizes and neutralizes both Stx and Stx2 in the HeLa cell cytotoxicity assay (Donohue-Rolfe *et al*., 1989a).

Furthermore, Bielaszewska *et al*. found that immunization of rabbits with chemically prepared Stx1 or Stx2 toxoids, or A subunit proteins of either toxin prevents the localization of heterologous toxin types to tissues targeted during systemic toxin-mediated disease. In that study, the B subunits of Stx1 or 2 did not provide heterologous protection (Bielaszewska *et al*., 1997). Additional evidence of cross-protection *in vivo* was reported by Ludwig *et al*. where protection against Stx1 challenge in rabbits was provided by a chemically prepared Stx2 toxoid immunization (Bielaszewska *et al*., 1997; Ludwig *et al*., 2002a). On the other hand, we and others have noted that rabbit polyclonal antiserum against one toxin type does not to neutralize the activity of the other toxin type in the Vero cell cytotoxicity assay (Strockbine *et al*., 1986; Downes *et al*., 1988; Gentry *et al*., 1980). Similarly, passive immunization of mice with anti-Stx2A monoclonal antibody protects mice from the effects of infection with Stx2-producing strains while anti-Stx1B monoclonal antibody is not protective against such a challenge (Wadolkowski *et al*., 1990; Lindgren *et al*., 1993). Additionally, a portion of humans who have been infected with Shiga toxin-producing *E. coli* develop specific antibodies to the Stxs produced by the infecting organism (Ludwig *et al*., 2002b), and the antibodies against Stx1 and Stx2 are distinct as assessed by Western blots. This latter finding suggests that in humans, cross-reactivity and cross-neutralization may not occur.

Resolution of the question of whether antibodies cross-neutralize Stx1 and Stx2 is a critical one for the design of a vaccine to protect against the severe toxin-mediated sequela of infection by Shiga toxin-producing *E. coli*. In this study, we sought to determine whether Shiga toxoid immunization is protective against heterologous toxin types in mice. For this purpose, genetic toxoids of Stx1 and Stx2 were constructed by

changing amino acids previously shown to be critical to the enzymatic activity of the A subunits. This genetic approach to toxoid production was elected because conventional chemical inactivation of toxin with formaldehyde or gluteraldehyde is an ill-defined chemical process that can result in residual toxicity (Metz *et al*., 2003; Gordon *et al*., 1992b). The potential for life-threatening toxicity of such a vaccine precludes the use of chemical Stx toxoids in humans. Second, chemical inactivation may distort the native holotoxin structure such that neutralizing antibodies are not generated or are of low titer. Third, genetic alteration of the enzymatic site preserves the general holotoxin structure to allow the development of antibodies against the entire toxin multimer. Finally, previous studies in our laboratory have shown that a genetic toxoid of Stx2e provides a safe holotoxoid vaccine that protects swine from edema disease (Gordon *et al*., 1992b). Therefore, we elected to employ the same strategy with Stx1 and Stx2.

Materials and methods

Construction of Stx1 and Stx2 toxoids

Mutations were generated in *stx1A* from pCKS112 (Tesh *et al*., 1993) with the Stratagene QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Primer pairs, 1A(Y77S)-forward and 1A(Y77S)-reverse, directed the change of tyrosine 77 to serine (Table 1). The change from glutamate to glutamine at position 167 was introduced by gene splicing by overlap extension (White 1993). The mutagenic primer pairs were 1A(E167Q)-forward and 1A(E167Q)-reverse (Table 1). The PCR primers 5'-GTGGATCCTCAAGGAGTATTG- 3' and 5' -GTGGATTCAACAACTGACTG- 3' were then used to generate and amplify the recombinant mutant *stx2*. The DNA fragments were digested with *Bam*HI and *Eco*RI (New England Biolabs, Beverly MA) and ligated into pSK+ (Stratagene). The resulting plasmid was named pSW09. The mutations were made in *stx2A* from pMJ100 (Weinstein *et al*., 1989) with the Stratagene QuikChange Site-directed Mutagenesis Kit. The tyrosine to serine change at amino acid 77 was made with primer pair 2A(Y77S)-forward and 2A(Y77S)-reverse (Table 1). The glutamate to glutamine change at position 167 was made with primer pair 1A(E167Q)-forward and 1A(E167Q)-reverse (Table 2). The resulting plasmid that contained both changes was named pNR100. Mutations were confirmed by DNA sequence determinations with the ABI Prism Big Dye Terminator Sequencing Kit version 3.2 (Applied Biosystems, Foster City, CA) at the Biomedical Instrumentation Center, of the Uniformed Services University. Loss of toxicity of the toxoids was assessed by Vero cell cytotoxicity assay (Gentry *et al*., 1980). Vero cells were challenged with 25 μg/ml of

Table 2. Oligonucleotide sets for mutagenesis in *stx1A* and *stx2A*

Primers	Sequence[a]
1A(Y77S)-forward	5'-CGGCTTATTGTTGAACGAAATAATTTATCTGTGACAGGATTTG- 3'
1A(Y77S)-reverse	5' -CAAATCCTGTCACAGATAAATTATTTCGTTCAACAATAAGCCG- 3'
1A(E167Q)-forward	5' -CGGTTTGTTACTGTGACAGCTCAAGCTTTACGTTTTCGGC- 3'
1A(E167Q)-reverse	5' -GCCGAAAACGTAAAGCTTGAGCTGTCACAGTAACAAACCG- 3'
2A(Y77S)-forward	5' -GATTATTGAGCAAAATAATTTATCTGTGGCCGGGTTCGTTAATACGG- 3'
2A(Y77S)-reverse	5'-CCGTATTAACGAACCCGGCCACAGATAAATTATTTTGCTCAATAATC- 3'
2A(E167Q)-forward	5' -GTTTTGTCACTGTCACAGCACAAGCCTTACGCTTCAGGCAG- 3'
2A(E167Q)-reverse	5' -CTGCCTGAAGCGTAAGGCTTGTGCTGTGACAGTGACAAAAC- 3'

[a]Sites of mutagenesis are underlined

toxoid, a protein concentration equivalent to 1.25×10^6 times 50% cytotoxic doses (CD_{50}) of native Shiga type toxins(Kaper *et al*., 1998b). The potential for residual *in vivo* toxicity was determined by lethality assays with intraperitoneal injection of toxoids in 2 groups of 5 BALB/c mice. Mice were given doses of toxoids equivalent to either 200 times the dose of toxin required to kill 50% of the animals (LD_{50}) for the wild-type Stx1, or 500 times the LD_{50} for Stx2 (40 μg for Stx1 and 1 μg for Stx2).

Purification of Stx1 and Stx2 toxoids

Stx1 and Stx2 toxoids were purified by affinity chromatography with commercially available Globotriose Fractogel (IsoSep AB, Tullinge, Sweden) as described by Ishikawa *et al*. (Ishikawa *et al*., 2003). Briefly, *E. coli* strain DH5α transformed with either pSW09 (for Stx1 toxoid) or pNR100 (for Stx2 toxoid) was grown overnight at 37°C in Luria-Bertani (Difco, Detroit, MI) broth that contained 100 μg per ml of ampicillin (Sigma, St. Louis, MO). The bacteria were sedimented by centrifugation, concentrated 500-fold, and then lysed by sonication. The lysate was clarified by centrifugation and 1 ml was applied to a 2-ml Globotriose Fractogel column. The column was incubated at 4°C for two hours and washed 3 times with 5 ml of phosphate-buffered saline (PBS) (pH7.4). Toxoid was then eluded from the column with 5 ml of 4 M $MgCl_2$ in PBS: this process was repeated three times. The eluded material was pooled, dialyzed in PBS, and concentrated by centrifugation filtration using the Centriplus system (Millipore, Bedford, MA) to a concentration of approximately 20μg of protein per ml. The concentrated toxoid preparation was examined for homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The presence of endotoxin in the

purified toxoid preparations was analyzed with the Pyrosate® Rapid Endotoxin Detection kit (Associates of Cape Cod, Inc., East Falmouth, MA) according to the manufacture's instructions.

Immunization, sample collection, and challenge

Groups of five female 3 to 4- week-old BALB/c mice each (Charles River Laboratories, Wilmington, MA) were immunized I.P. on day zero and 21 days later with 1 μg total of Stx1 toxoid, Stx2 toxoid, an equal mixture of both toxoids, or PBS, each mixed with an equal volume of TiterMax® Gold (TiterMax USA, Inc., Norcross, GA; www.titermax.com), a water-in-oil adjuvant. Serum was collected from the tail vein of each animal prior to and 28 days after the first immunization. On day 35 after the initial immunization, mice were challenged I.P. with a mixture of 10 μg of Stx1 and 100 ng of Stx2, the equivalent of 50 times the LD_{50} of each wild-type toxin (Ishikawa *et al*., 2003; Tesh *et al*., 1993).

Analysis of antibody response

Toxin-specific IgG in serum was analyzed by immunoblot. Purified wild-type Stx1 or Stx2 was subjected to SDS-PAGE, and electrotransferred to nitrocellulose. The blots were then probed with mouse serum diluted 1:100 followed by horseradish peroxidase-conjugated secondary rabbit antibodies against mouse IgG (Bio-Rad Laboratories, Hercules, CA).

Neutralization of cytotoxicity of Stx1 and Stx2

Toxin-neutralizing antibody was detected in mouse serum through inhibition of Shiga cytotoxicity in the Vero cell cytotoxicity assay. Vero cells were grown overnight to confluency in 96-well plates with Eagle's minimum essential medium (EMEM) (Cambrex Bio Science, Inc., Walkersville, MD) supplemented with 10% (vol/vol) fetal bovine serum, 0.8 mM glutamine, 500 U of penicillin G per ml, and 500 μg of streptomycin per ml. Serially diluted sera from immunized mice were incubated at 37°C for 1 hour with standard concentrations of 0.2 ng/ml each of the wild-type Stx1 or Stx2 diluted in EMEM supplemented as above. This concentration correlated to 10 times the CD_{50} for each toxin (Kaper *et al*., 1998b). Each serum-toxin mixture was then overlaid onto the cultured Vero cells, and the plates were incubated for 48 hours at 37^{o}C and 5% CO_2. The microtiter plates were fixed in formalin, stained with crystal violet, and absorbance measured at 600 nm. The neutralization titer was defined as the reciprocal of the highest dilution of post-immune serum that protected Vero cells as determined by optical densities greater than those seen with the toxin alone, or toxin mixed with pre-immune mouse serum.

Statistical analysis

The level of toxin-neutralizing antibody detected in each toxoid immunization group was reported as the geometric mean of the neutralization titers of the five mice in each group (plus and minus one standard error of the mean). The degree of protection afforded by immunization with one or both Stx toxoids was determined from the number of mice that survived toxin challenge. Ten days post challenge the numbers of mice that

survived single versus dual toxin administration were compared and the significance of these differences determined by Fisher's exact test.

Results

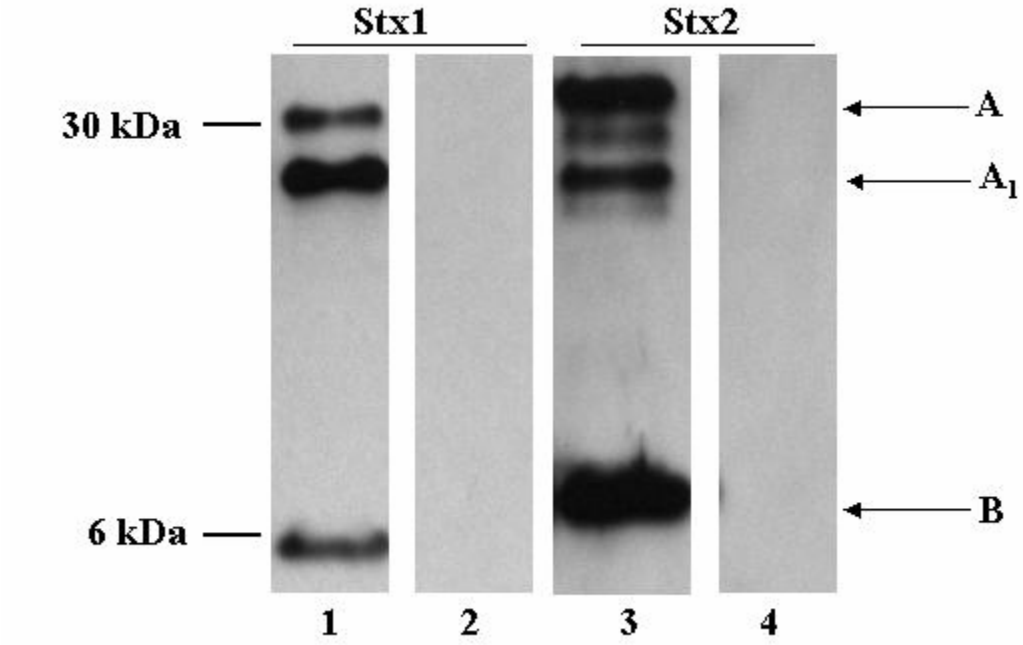
Construction, purification, and toxicity assessment of Stx1 and Stx2 toxoids

The glutamic acid at position 167 of the A subunit is the active site and is critical for enzymatic activity for both Stx1 and Stx2 (Hovde *et al*., 1988; Jackson *et al*., 1990; Gordon *et al*., 1992b); therefore, it was a target for inactivation by site-directed mutagenesis. To avoid spontaneous reversion to toxicity, another amino acid residue in the A subunit that is also important for toxicity, the tyrosine at position 77, was also changed (Deresiewicz *et al*., 1992). To assess whether these mutations had indeed rendered the resultant proteins non-toxic, both toxoids were tested for cytotoxicity to Vero cells. Neither toxoid showed cytotoxic effects even when the Vero cells were treated with protein concentrations equivalent to 1.25×10^6 times the CD_{50} of native toxin. Similarly, mice treated intraperitoneally with toxoids with protein concentrations equivalent to between 200 and 500 times the 50% lethal doses of Stx1 and Stx2 showed no ill effects. We concluded that the alteration of the two amino acids in each of the toxins had completely abrogated toxic activity as assessed in these tissue culture and animal assays.

Next, purified toxoid preparations were assessed following PAGE by silver stain and Western blot with rabbit anti-Stx1 or anti-Stx2 polyclonal antibodies (Strockbine *et al*., 1986) (Fig. 9). The sliver stain demonstrated that the toxoid preparations contained very little contaminating protein (data not shown). The identity of the putative toxoid proteins seen on Silver stain was verified by Western blot (Fig. 9). Additional bands were recognized by the polyclonal antisera against the A subunits of the toxoids. We

Figure 9. Western blots of purified toxoids.

Purified Stx1 and Stx2 toxoids (1 μg) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with either rabbit polyclonal anti-Stx1 or anti-Stx2 sera followed by horseradish peroxidase-conjugated goat anti-rabbit antibody. Lanes 1 and 3 represent toxoids probed with the homologous antisera and lanes 2 and 4 represent toxoids probed with heterologous antisera. The A, A_1 and B subunit positions are indicated by arrows.


Stx1
Stx2
30 kDa
6 kDa
A
A1
B
1
2
3
4

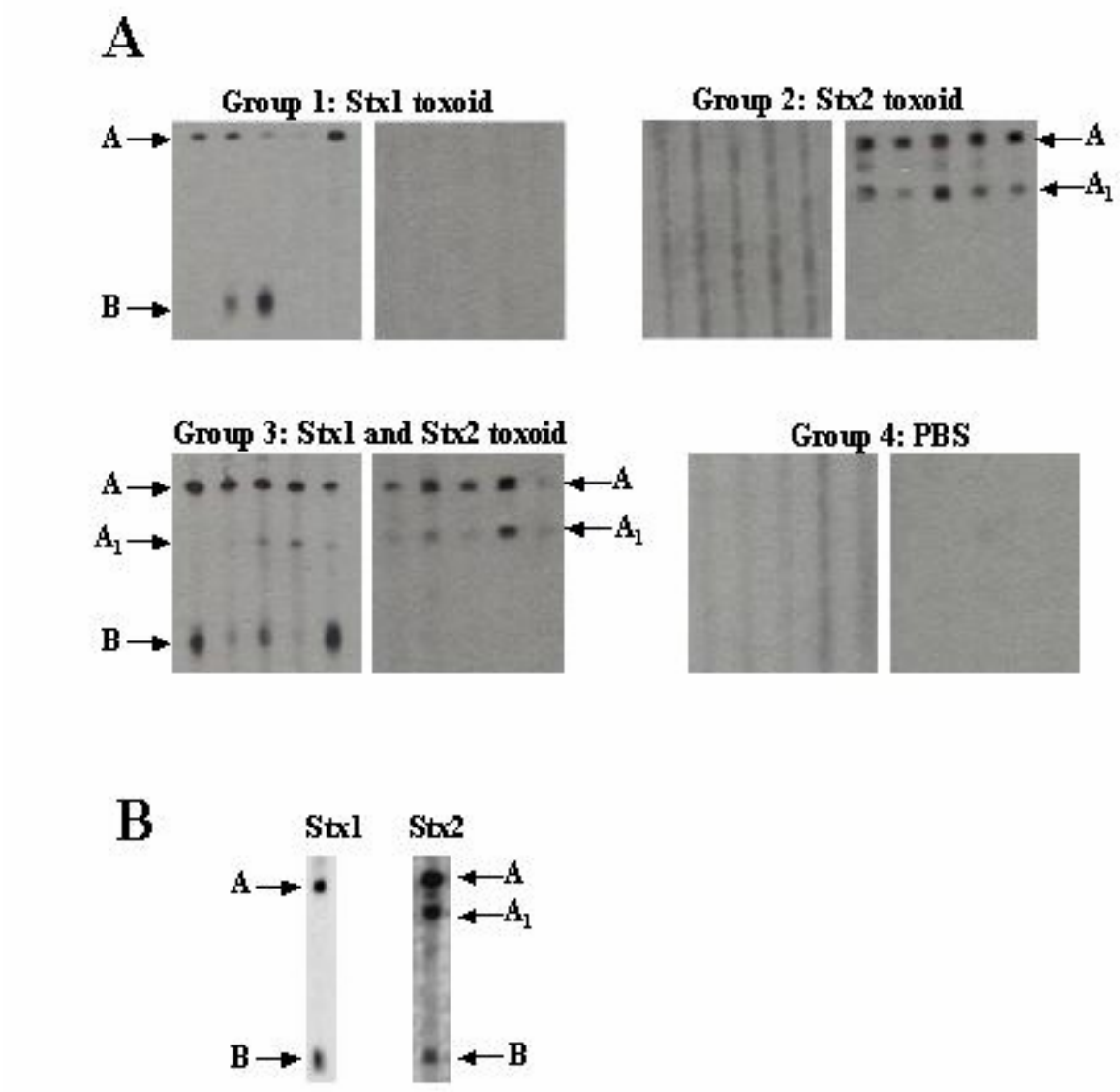
speculated that these bands represented the reduction of the A chain to yield both full length A and A1 as well as degradation products of Stx2. The preparations were also tested for the presence of endotoxin and were shown to contain endotoxin at a concentration greater than 0.25 EU/ml (0.25 EU/ml is the limit of detection for the agglutination assay). This finding was expected because the toxoids were purified from culture sonicates of *E. coli* transformed with plasmids encoding the toxoid genes. Nonetheless, the toxoid preparations appeared non-toxic to mice, as noted above.

Toxin-specific antibody response

Because the most serious complications of Shiga intoxication are systemic, we focused on the detection of IgG type antibodies against Stx1 and Stx2 in the sera of immunized mice. The antisera from the Stx1 toxoid-immunized mice reacted only to Stx1 on the Western blot (Fig. 10A, Group 1), and those immunized with Stx2 toxoid reacted only with Stx2 (Fig. 10A, Group 2). The antisera from mice immunized with both toxoids reacted with both the Stx1A and B subunits and the Stx2A subunit by Western blot (Fig. 10A, Group 3). The Stx2B subunit was not detected with mouse antiserum raised against Stx2 toxoid. To verify that both the A and B subunits of Stx1 and Stx2 were present on the blot, Western blots of the toxins were probed with rabbit polyclonal antibodies against each homologous toxin type. The respective rabbit polyclonal antisera detected both the A and B subunits of each toxin (Fig. 10B). One explanation for the failure of mouse serum to detect the B subunit of Stx2 is that Stx2B is less immuno-reactive than Stx1B and was not detected by antibodies of low

Figure 10. Immunized mice produced serum IgG to the homologous toxin.

(A) The immunogen administered is indicated at the top of each panel. Purified wild-type Stx1 (left blot in each panel) and Stx2 (right blot in each panel) were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Each lane was probed with serum from an individual mouse followed by horseradish peroxidase-conjugated rabbit secondary antibodies against mouse IgG. The results are representative of two independent experiments with five mice per group. (B) Positive controls to demonstrate that A and B subunits of each toxin are present as indicated by reactivity with the homologous rabbit polyclonal antisera. The A, A_1 and B subunit positions are indicated by arrows.


A
Group 1: Stx1 toxoid
A
B
Group 2: Stx2 toxoid
A
A1
Group 3: Stx1 and Stx2 toxoid
A
A1
B
A
A1
Group 4: PBS
B
Stx1
Stx2
A
B
A
A1
B

concentration. Another explanation is that Stx2B is less immunogenic than Stx2A, and most of the antibodies elicited in mice were directed to the immuno-dominant A subunit.

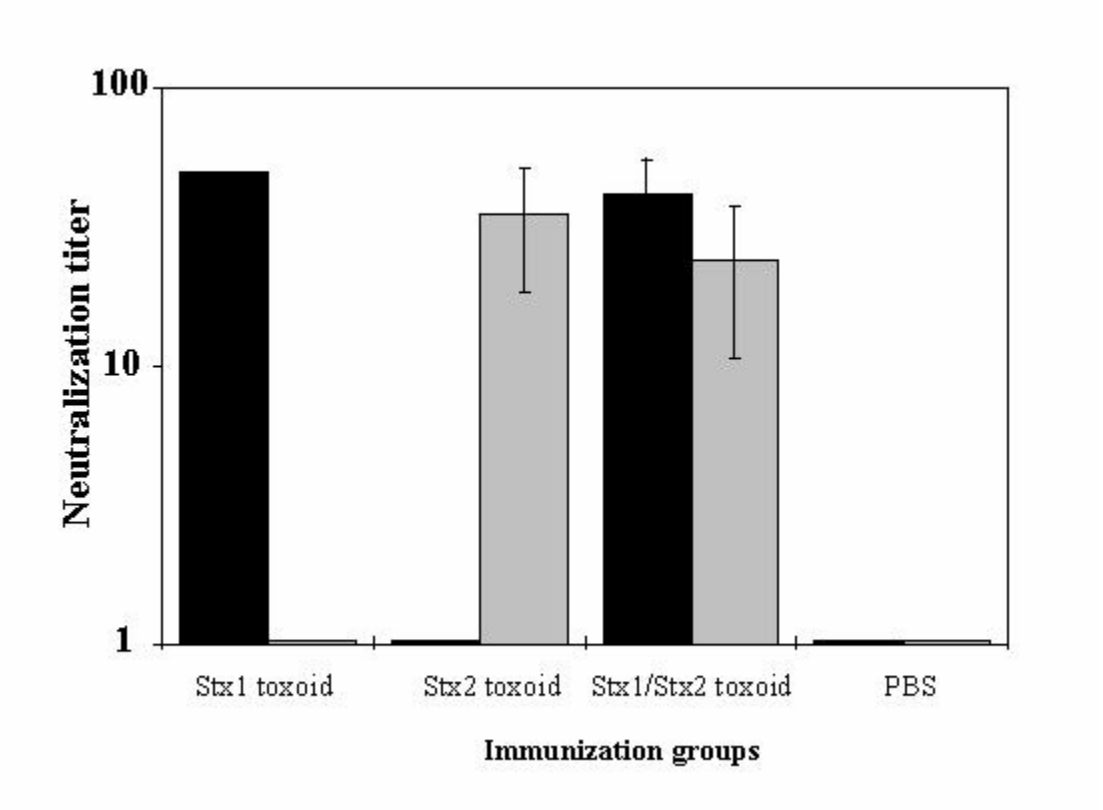
Not all the serum samples from the mice in the Stx1-immunized group showed anti-Stx1B antibodies on Western blot; however, serum from each mouse in the group immunized with both toxoids reacted with Stx1B and Stx2B on Western blots. These findings suggested that the anti-Stx1B antibody response was enhanced when both toxoids were present in the vaccine. Serum samples from the Stx1 toxoid-immunized group all had similar levels of toxin-neutralizing activity, regardless of whether they contained detectable anti-Stx1B polypeptide antibodies by Western blot. This latter observation suggests that the antibodies to the B subunit are not required for toxin neutralization.

Neutralization of Stx1 and Stx2

The production of antibodies that neutralize the cytotoxicity of Shiga toxins to Vero cells by toxoid-immunized mice was considered an *in vitro* indicator of a protective immune response. Neutralization titers are depicted in Figure 11. Antisera from mice immunized with Stx1 toxoid neutralized only Stx1 *in vitro*, and antisera from mice immunized with Stx2 toxoid neutralized only Stx2 *in vitro*. The mice immunized with both toxoids produced antibodies that neutralized both Stx1 and Stx2 in the Vero cell assay. We did not observe any mice that produced antibodies that reacted with toxin by Western blot but did not neutralize toxin in the Vero cell assay. Similarly, none of the sera provided a neutralizing effect on Vero cells without recognition of the specific toxin type by Western blot. These observations suggested that the holotoxoids would be viable

Figure 11. Neutralization of Stx1 and Stx2 Vero cytotoxicity with mouse antisera.

Serum collected from each mouse per immunization group was serially diluted and pre-incubated with either purified wild-type Stx1 (■) or Stx2 (■) equivalent to ten 50% cytotoxic doses. The mixture was then applied to Vero cells. The neutralization titer was defined as the reciprocal of the highest dilution of post-immune serum that gave an absorbance reading above both pre-immune and toxin-only control wells. The data are expressed as the geometric mean of neutralization titers obtained from five mice per group +/- one standard deviation. The dashed line represents the limit of detection. The results represent two independent experiments with five mice per group.


100
10
1
Neutralization titer
Stx1 toxoid
Stx2 toxoid
Stx1/Stx2 toxoid
PBS
Immunization groups

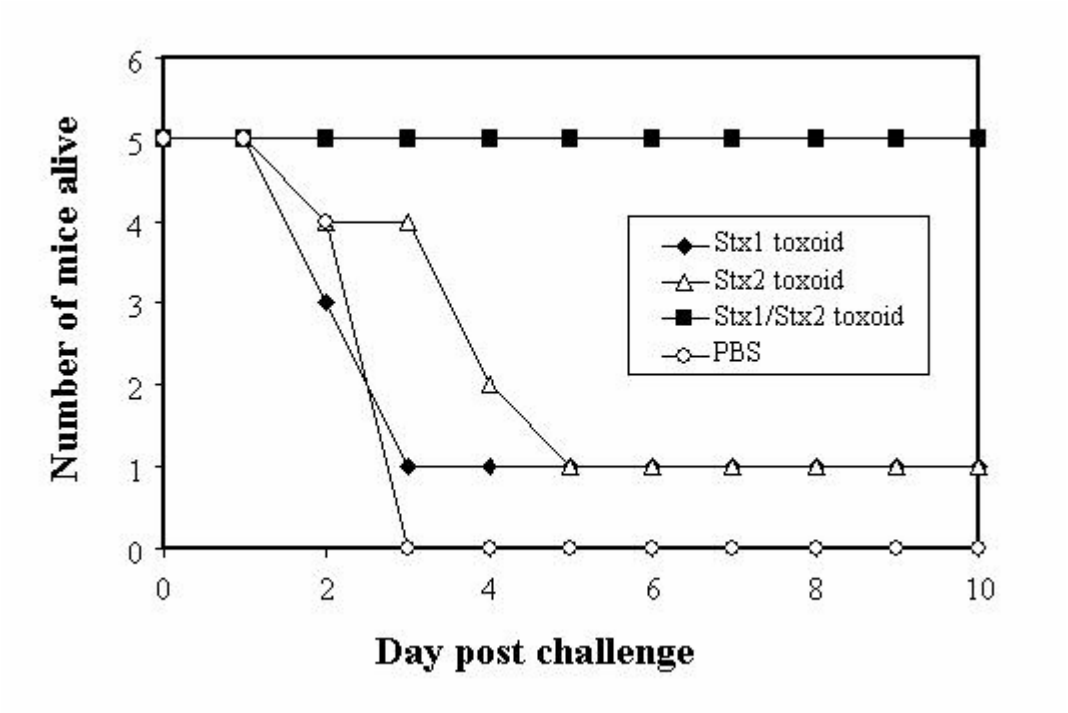
candidates for vaccine development because they induced toxin-neutralizing antibody. Rabbit antisera against Stx1 or Stx2 served as controls in the neutralization assay and no cross-neutralization was observed (data not shown).

Protection from dual-toxin challenge

On day 35 after the initial immunization, all mice were challenged I.P. with a mixture of purified wild-type Stx1 and Stx2 equivalent to 50 LD_{50}s each. The mice immunized with both toxoids were fully protected, whereas mice immunized with only one toxoid did not survive the challenge (Figure 12). This lack of cross-protection indicated that both toxoids were needed to protect animals from a multi-toxin challenge. The dual-toxin challenge results correlated with the Western blot and toxin neutralization studies, and confirmed that *in vivo* the anti-toxoid antibodies were type specific and not cross-protective.

Figure 12. Survival of mice following dual-toxin challenge.

Mice were injected I.P. with a mixture of $50LD_{50}$ each of purified wild-type Stx1 and Stx2 (10 μg Stx1; 100 ng Stx2) on day 35 post initial immunization. Statistical analysis was done on day 10 to compare single-toxoid-immunized groups with the double-toxoid-immunized group using Fisher's exact test ($p < 0.05$). The results represent two independent experiments with five mice per group.


Number of mice alive
Day post challenge
Stx1 toxoid
Stx2 toxoid
Stx1/Stx2 toxoid
PBS
0
1
2
3
4
5
6
0
2
4
6
8
10

Chapter Three

A plant-based oral vaccine to protect against systemic Shiga toxin type 2 intoxication

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Note: all of the figures and tables shown reflect the work of Sharon Wen with the exception of the plant-optimization of stx_2 performed by Dr. Nicole Judge. Drs. Teel and O'Brien contributed to the design of the experiments and interpretation of the data as well as the preparation of the manuscript. The order of this chapter (Abstract, Introduction, Results, and Material and Methods) is in accordance with the submission requirements posed by *Proceedings of the National Academy of Sciences*.

Abstract

Hemolytic uremic syndrome (HUS), the leading cause of kidney failure in children, often follows infection with Enterohemorrhagic *Escherichia coli* and is mediated by the Shiga type toxins, particularly type 2 (Stx2) produced by such strains. The challenge in protecting against the life-threatening HUS is to stimulate an immune response at the site of infection that also protects against Shiga intoxication at distal sites such as the kidney. As one approach to meet this challenge, we sought to develop and characterize a prototypic orally-delivered, plant-based vaccine against Stx2, an AB_5 toxin. First, we genetically inactivated the Stx2 active A subunit gene and then optimized both subunit genes for expression in plants. Then, the toxoid genes were transformed into the *Nicotiana tabacum* (tobacco) cell line, NT-1, via *Agrobacterium tumefaciens*-mediated transformation. Toxoid expression was detected in NT-1 cell extracts and the assembly of the holotoxoid was confirmed. Finally, mice were immunized by feeding the toxoid-expressing NT-1 cells or by parenteral immunization followed by oral vaccination (prime-boost strategy). The immunized mice produced Stx2-specific mucosal IgA and Stx2-neutralizing serum IgG. The protective efficacy of these responses was assessed by challenging the immunized mice with *E. coli* O91:H21 strain B2F1, an isolate that produces an activatable variant of Stx2 (Stx2d) and is lethal to mice. The oral immunization fully protected mice from the challenge. To the best of our knowledge, this was the first demonstration that a plant-based, oral vaccine can confer protection against lethal systemic intoxication.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) cause an estimated 100,000 cases of hemorrhagic colitis per year in the United States (Mead *et al*., 1999). Disease is most often associated with enterohemorrhagic *E*. *coli* (EHEC), a subset of STEC that includes serotype O157:H7. Six percent of these infections progress to the life-threatening systemic disease called the hemolytic uremic syndrome (HUS) that is characterized by thrombocytopenia, hemolytic uremia, and kidney failure. HUS occurs as a consequence of the action of the members of the Shiga toxin (Stx) family (Griffin *et al*., 1991). Stxs are AB_5 holotoxins that consist of one catalytic A subunit, a 28S rRNA N-glycosidase polypeptide (Furutani *et al*., 1990), that is non-covalently associated with a pentameric B subunit that binds to the widely distributed globotriaosyl ceramide (Gb_3) cellular receptor (Donohue-Rolfe *et al*., 1989b; Jacewicz *et al*., 1986). Two major types of Stx are produced by STEC that infect humans, Stx1 and Stx2. They are only 55% identical by amino acid sequence and consequently are antigenically distinct. Antibodies raised against one type do not cross-neutralize the other (Strockbine *et al*., 1986; Downes *et al*., 1988; Wadolkowski *et al*., 1990). Although STEC strains may produce one or both types of Stx, Stx2 is associated with the most severe disease in humans. Therefore, we began our efforts to develop a vaccine against Stx intoxication by focusing on Stx2.

Plant-based vaccines have several advantages: 1) they provide a convenient, safe, and familiar vehicle for oral delivery (Tacket 2004); 2) they are inexpensive to produce in large scale settings (Streatfield *et al*., 2003); 3) they are unlikely to be contaminated with animal pathogens, a potential problem with tissue culture-derived vaccines (Cimolai

2001); and, 4) encapsulation of immunizing proteins in plant tissue protects the antigens destined for delivery to the gut (Streatfield 2005). We recently described the construction of an orally-delivered, plant-based vaccine against the primary adherence factor of EHEC, intimin. In that study, the *Nicotiana tabacum* cell line NT-1 was modified via *Agrobacterium*-mediated transformation to express the C-terminal binding domain of intimin. Upon oral challenge with a wild-type EHEC strain, colonization was reduced in the mice that had been fed a regime of intimin-expressing NT-1 cells (Judge *et al*., 2004).

In the work described here, we used the same strategy to express a genetically inactivated Stx2 toxoid in NT-1 cells to stimulate an immune response in the gut against Stx2, and, more importantly, to protect against the systemic Stx2 intoxication. The efficacy of the vaccine was tested in the streptomycin-treated mouse model of STEC infection. In that model, oral challenge with an *E. coli* O91:H21 strain B2F1 that produces an activatable variant of Stx2 (Stx2d) results in tubular necrosis and death (Ito *et al*., 1990; Lindgren *et al*., 1993). That this toxin is entirely responsible for kidney lesions and death of infected mice is indicated by the repeated finding that with passive administration of neutralizing monoclonal anti-Stx2 antibodies the infected animals survive with no evidence of renal pathology (Melton-Celsa *et al*., 1998; Edwards *et al*., 1998).

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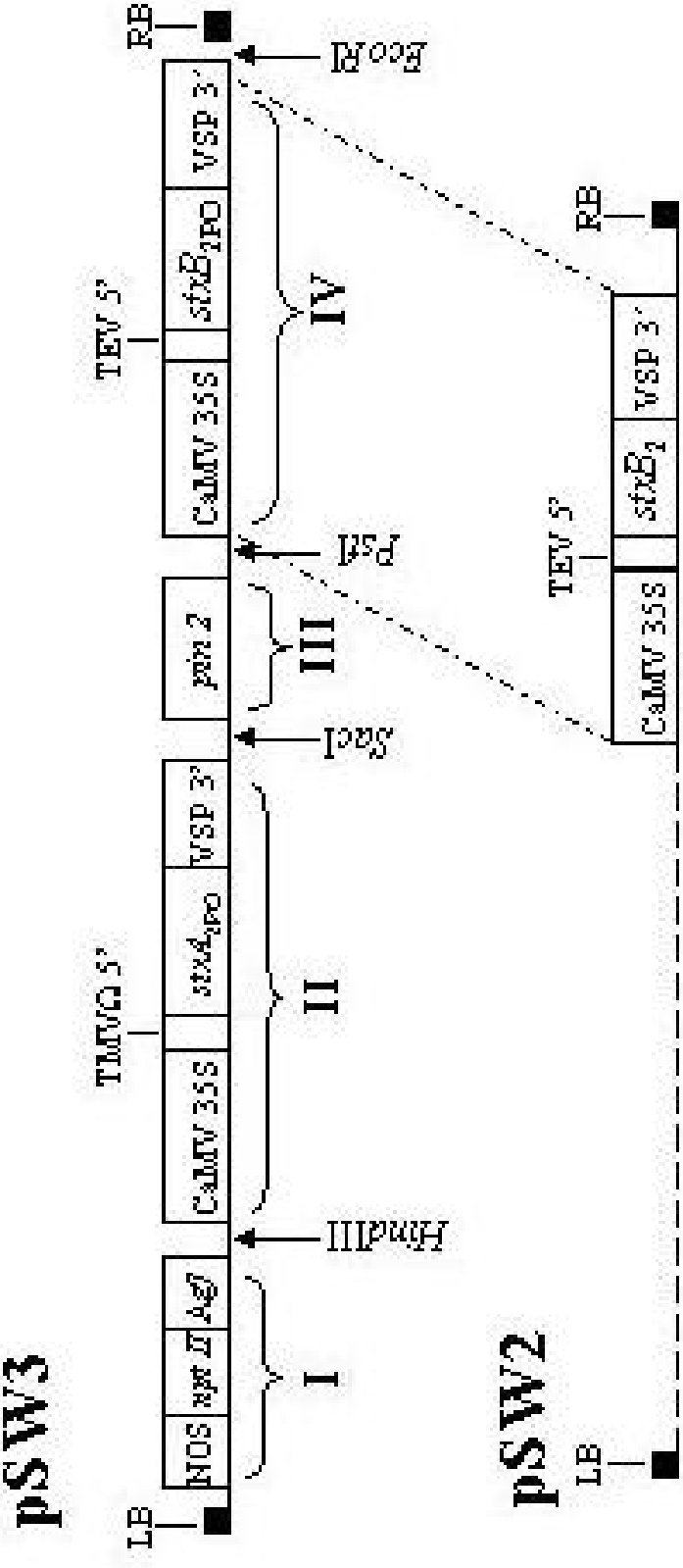
Results

Expression of Stx2 genes in NT-1 cells

First, the bacterial Stx2 toxoid-encoding sequences were modified, or plant-optimized (PO), to eliminate sequences that might be recognized as eukaryotic mRNA termination sites, splice sites, or polyadenylation sequences in NT-1 cells and thus alter antigen expression (Adang *et al*., 1993; Perlak *et al*., 1991; Newman *et al*., 1993). Three such silent mutations were introduced into the toxoid A subunit to yield $stxA_{2PO}$. A plant-optimized $stxB_2$ gene ($stxB_{2PO}$) was synthesized by ligase chain reaction (LCR) to incorporate a total of 37 silent mutations. Next, the modified StxA2 and B2 subunit genes, which are transcribed in one operon by bacteria, were cloned as individual open reading frames flanked with plant or viral elements to facilitate transcription of each protein in NT-1 cells (Fig. 13). Thirdly, two dual-expression cassettes were assembled. One consisted of the plant-optimized Stx2A toxoid gene and the plant-optimized StxB2 gene separated by a terminator sequence to insure the independent expression of both toxoid subunits. The other cassette contained the plant-optimized Stx2A toxoid gene, the intergenic terminator, and the native StxB2 gene. Finally, each dual expression cassette was cloned into the binary vector pGPTV-Kan (Becker *et al*., 1992) between the left and right border sequences that define the region that is transferred to plants (T-DNA) during *Agrobacterium* infection. The resulting plasmids, pSW2 (toxoided $stxA_{2PO}$/native $stxB_2$) or pSW3 (toxoided $stxA_{2PO}$/ $stxB_{2PO}$), were transformed into *A. tumifaciens* that was then used to infect NT-1 cells as described previously (Judge *et al*., 2004).

Figure 13. Diagram of the plant transformation vectors pSW3 and pSW2.

Genetic elements in pGPTV-Kan between the left and right border sequences (LB and RB) that define the T-DNA were; **I**: *npt II*, the neomycin phosphotransferase gene for kanamycin resistance flanked by the nopline synthase promoter (NOS) and the nopline synthase polyadenylation signal (Ag7); **II:** the toxoid $stxA_{2PO}$ flanked by the cauliflower mosaic virus 35S promoter (CaMV) followed by the 5'untranslated region (UTR) from tobacco mosaic virus (TMVΩ) and the soybean vegetative storage protein 3' polyadenylation sequence; **III:** an intergenic terminator sequence derived from the potato proteinase inhibitor II gene, *pin2*; **IV:** the plant-optimized $stxB_{2PO}$ (pSW3) or native $stxB_2$ (pSW2) eukaryotic expression cassette with the tobacco etch virus (TEV) 5' UTR. Restriction sites used for ligations are indicated below the map.

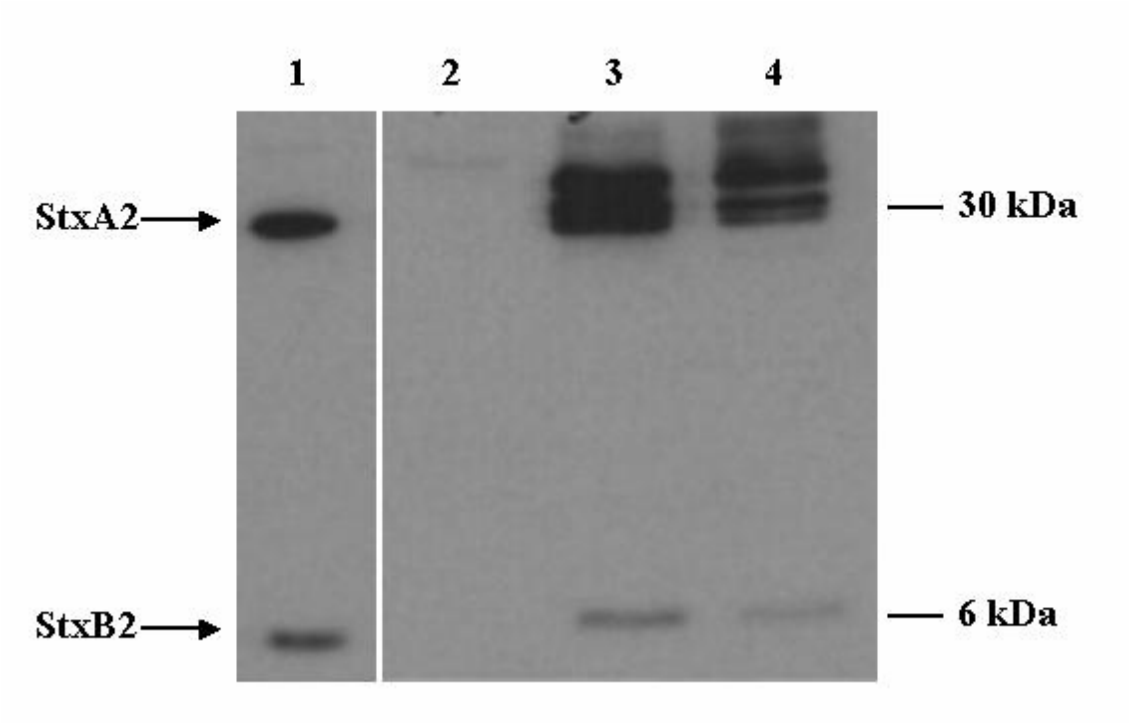

pSW3
LB
NOS
nptII
AgtI
CaMV 35S
TMVΩ 5'
stx4pro
WSP 3'
pin 2
SacI
PsiA
CaMV 35S
TEV 5'
stx2pro
WSP 3'
EcoRI
RB
HindIII
I
II
III
IV
pSW2
LB
CaMV 35S
TEV 5'
stx2
WSP 3'
RB

Since *Agrobacterium*-mediated transformation results in random insertions of the T-DNA into plant chromosomes, the levels of expression of transferred genes may vary among recipient clones depending on the site of insertion. Western blot analysis was used to identify kanamycin-resistant transformants that expressed detectable levels of Stx2 toxoid (Fig. 14). Ten of the 17 kanamycin-resistant NT-1 clones derived from pSW2 and six of the 10 derived from pSW3 yielded detectable Stx2 expression by Western blot analysis (data not shown). Two representative clones, NT#2 transformed with the $stxA_{2PO}/stxB_2$, and NT#3 transformed with $stxA_{2PO}/stxB_{2PO}$ toxoid genes, were selected for further studies because they maintained stable toxoid expression. Another NT-1 clone that was transformed with the pGPTV-Kan vector that encodes the kanamycin resistance marker *nptII*, but no other open reading frames in the T-DNA region, was renamed NT-1Kan^R and served as negative vaccine control material.

The antigen concentrations expressed by NT-1 cells were estimated from Western blots by comparison of pixel densities of bands observed with known concentrations of purified Stx2 with those observed from NT-1 cell extracts. The NT#2 clone yielded 8.2 +/- 0.5 μg of Stx2 toxoid per gram of tobacco cells and NT#3 yielded 6.5 +/- 2.5 μg per gram of NT-1 cells. The sequence changes in the plant optimized B subunit gene did not seem to improve protein expression; however, the chromosomal sites of toxoid gene insertion may also have influenced the relative levels of toxoid expression in these two candidate clones. We chose to continue the immunization studies with the fully plant-optimized clone NT#3 with the consideration that toxoid expression in other plant types that we plan to use in the future may require such optimization.

Figure 14. Western blot analysis of the Stx2 holotoxoid expressed by transformed NT-1 clones.

The positive control consisted of 10 ng of the wild-type Stx2 purified from *E. coli* DH5α that harbored pMJ100 (Weinstein *et al*., 1989) (lane 1). Sonic lysates of 50 mg of fresh plant cells were used to load lanes 2-4: lane 2, NT-1Kan^R clone served as the negative control; lane 3, NT#2 clone; and lane 4, NT#3 clone. Stx2 A and B subunit positions are indicated by arrows.


1
2
3
4
StxA2
StxB2
30 kDa
6 kDa

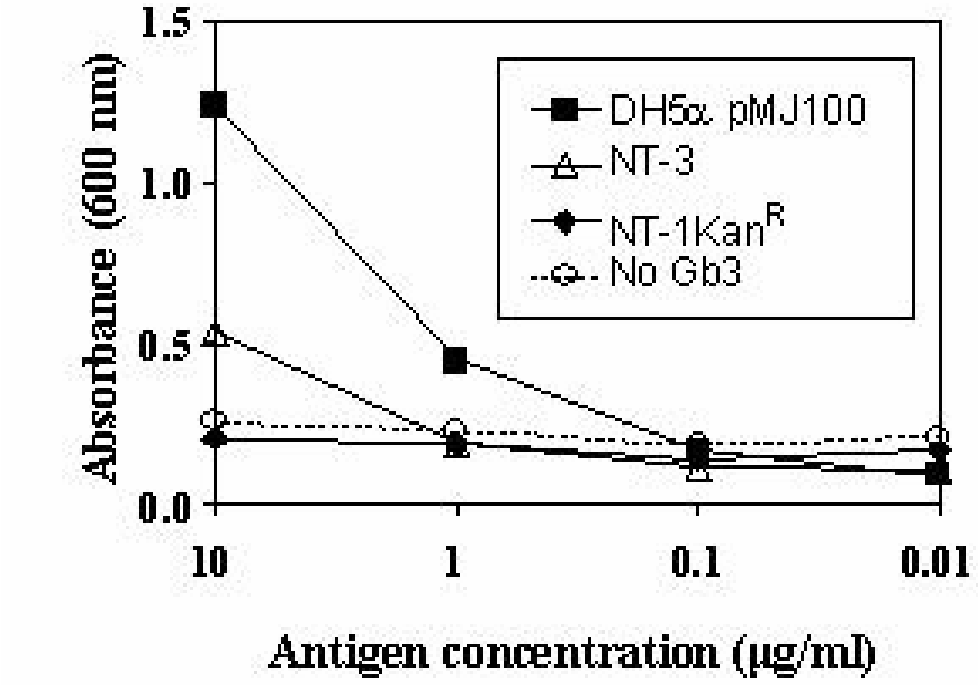
A Gb_3-based Enzyme-linked immunosorbent assay (ELISA) was used to determine if the plant-derived toxoid subunits assembled into the native holotoxin configuration. Since the N-terminus of the A subunit is critical for pentamerization of the B subunits (Haddad *et al*., 1993) and the B pentamer of Stx binds Gb_3 (Ashkenazi *et al*., 1989), antibody specific to the A subunit of Stx2 was used to verify the binding of both toxin components to Gb_3. We observed a positive reaction in a dose-dependent fashion with lysates from NT#3 (Fig. 15). From that finding we concluded that the tobacco cell-expressed A and B subunits were associated.

Immunogenicity of plant-derived toxoid

To first assess whether the plant-derived toxoid was functionally immunogenic against Stx2, we compared the immune responses in mice immunized intraperitoneally with either the plant-derived toxoid or a plasmid-encoded, *E. coli*-derived toxoid known to be protective against Stx2 (Wen *et al*., 2005). The geometric mean titers of Stx2-neutralizing antibody from animals immunized with plant- or *E. coli*-derived toxoid were 46 and 356, respectively. Despite this near log difference, the titers from these intraperitoneally- immunized mice were significantly higher than those of groups mock-immunized with NT-1Kan^R or *E. coli* that carried the pBluescribe vector alone. Furthermore, 10 of 10 mice immunized with the plant-derived toxoid and eight of eight mice immunized with *E. coli*-derived toxoid survived a parenteral challenge of 10 times the 50% lethal dose (10 LD_{50}) of Stx2. None of the mock-immunized animals survived such a challenge.

Figure 15. Assessment of plant-derived Stx2 holotoxoid assembly by the Gb_3-binding assay.

Serially diluted sonic lysates of *E. coli* DH5α (pMJ100) that expressed wild-type Stx2, NT#3, or NT-1Kan^R were added to Gb_3-coated wells. Wells without Gb_3 were also treated with *E. coli* DH5α (pMJ100) to serve as controls for non-specific binding by Stx2. The experiment was done in triplicate, and each datum point represents the mean value of replicates ± one standard deviation.


1.5
1.0
0.5
0.0
Absorbance (600 nm)
DH5α pMJ100
NT-3
NT-1Kan^R
No Gb3
10
1
0.1
0.01
Antigen concentration (µg/ml)

Oral immunization with transgenic tobacco cells

Preliminary studies were done to determine the optimal NT-1 toxoid dose, feeding conditions, and boosting schedule. Sera collected following these different vaccination regimes were assessed by ELISA to detect Stx2-specific IgG (data not shown). The vaccination strategy A (Table 3) in which mice were fed plant material once per week for five weeks was found to consistently elicit anti-Stx2 antibody and, therefore, was adopted for the remaining experiments. In addition, a prime-boost strategy that combined an initial intraperitoneal (I.P.) immunization with plant extract, followed by two oral feedings of NT-1 cells that expressed Stx2 toxoid (Group C) was adopted based on the success of such a strategy in eliciting an improved response to EHEC intimin and the heat-labile enterotoxin B subunit (LT-B) of enterotoxigenic *E. coli* in similar studies (Lauterslager *et al*., 2001; Judge *et al*., 2004).

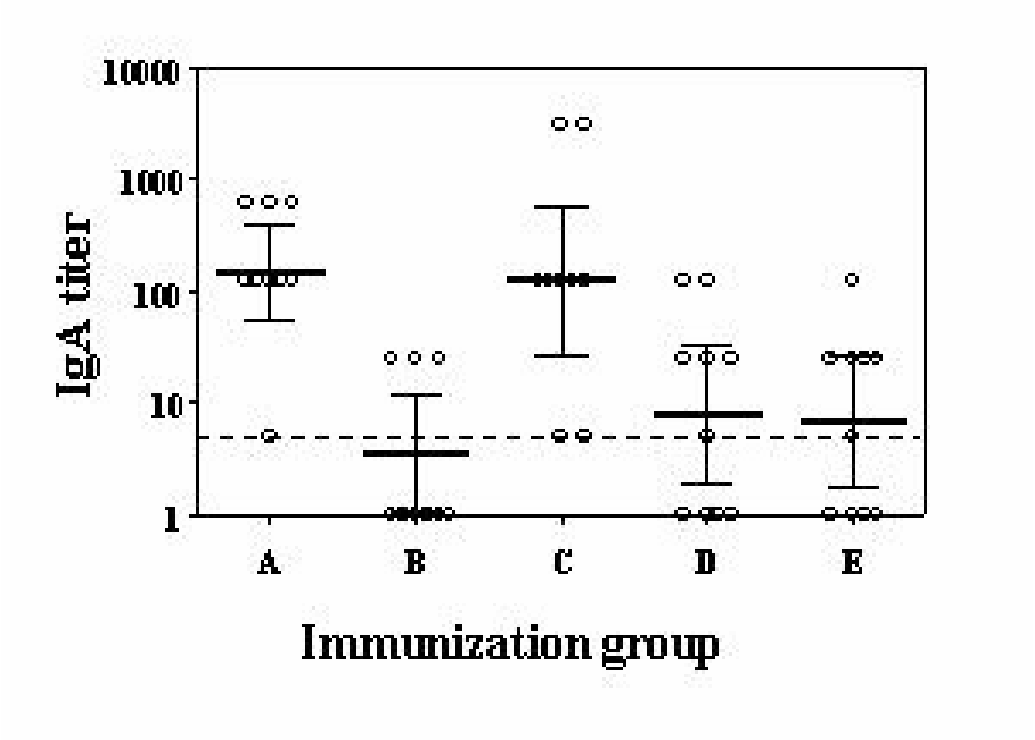
Fecal anti-Stx2 IgA was detected by ELISA. The geometric mean IgA titer of group A was statistically significantly higher than that of the mock-immunized group B (Fig. 16), and all but one mouse in Group A produced detectable anti-Stx2 IgA. We concluded that elevated fecal IgA titers in the immunized mice reflected a mucosal immune response. Though the geometric mean IgA titer of group C (parenterally- primed and orally boosted) was not statistically significantly higher then the geometric mean IgA titers of groups D (parenterally-primed and mock oral-boosted) and E (mock-primed and mock-boosted), the observed differences were noticeable. Furthermore, all the mice in group C produced detectable levels of anti-Stx2 IgA, whereas only 6 out of 10 mice in group D did. No difference was observed between groups D and E; a finding that

Table 3. Oral immunization groups and protocols

Groups	Immunization Protocol
A, Fed NT-Stx2 cells only	5 g of NT#3 cells fed on days 0, 7, 14, 21, and 28
B, Fed NT-1Kan^R cells	5 g of NT-1 vector-transformed cells fed on days 0, 7, 14, 21, and 28
C, Prime-boost with NT-Stx2 cells	Sonic lysate from 0.5 g of NT#3 + TiterMax I.P. on day 0; fed 5 g of NT-Stx2 cells fed on days 21 and 28
D, Prime-mock boost	Sonic lysate from 0.5 g of NT#3 + TiterMax I.P. on day 0; fed 5 g of NT-1Kan^R cells fed on days 21 and 28
E, Prime-boost with NT-1Kan^R cells	Sonic lysate from 0.5 g of NT-1Kan^R + TiterMax I.P. on day 0; 5 g of NT-1Kan^R cells fed on days 21 and 28

Figure 16. Stx2 specific fecal IgA as measured by ELISA.

Each circle represents the IgA titer of an individual mouse; the bar represents the geometric mean of the titers obtained from the group. The dashed line indicates the limit of detection and the error bracket represents the 95% confidence interval. Unpaired *t*-tests were performed between group pairs (see Table 1) A-B ($p < 0.05$), C-D ($p > 0.05$), C-E ($p > 0.05$), and D-E ($p > 0.05$).


1000
1000
100
10
1
IgA titer
A
B
C
D
E
Immunization group

suggested that I.P. priming did not enhance development of a mucosal immune response. Of note, both groups B and E were immunized with NT-1Kan^R only yet some mice in these groups showed anti-Stx2 fecal antibodies. Although purified Stx2 was used to coat the microtiter plates, it is not unusual to have some residual *E. coli* products in such toxin preparations (Wen *et al*., 2005). We suspect that the mock-immunized mice had antibodies against components of *E. coli* found among the gut flora that recognized such antigens in the purified recombinant Stx2 and that those antibodies contributed to background titers in these ELISAs.

A more correlative *in vitro* indicator of the protective immune response is the Vero cell cytotoxicity neutralization assay. The production of Stx2-neutralizing antibodies was assessed by incubation of mouse sera with wild-type Stx2 and the subsequent neutralization of Vero cell cytotoxicity by Stx2-specific antibodies in immune sera (Fig. 17). None of the mock orally-immunized mice produced detectable Stx2-neutralizing antibodies, whereas all of the orally immunized mice showed evidence of toxin-neutralizing antibodies. Furthermore, all of the mice in Group A were responders. The prime-boost method was also effective in eliciting production of neutralizing antibodies. The significantly higher neutralization titer of group C (I.P-primed and orally boosted) compared to group D (I.P.-primed and mock orally boosted) showed that oral feeding was important in eliciting the systemic response. Nonetheless, those that received only the parenteral plant extract vaccine (Group D) elicited a statistically significant neutralization titer compared to the mock immunization (Group E) ($p < 0.05$).

Figure 17. Neutralization of Stx2 Vero cytotoxicity with mouse anti-sera.

Each circle represents the neutralization titer of the serum from an individual mouse. The dashed line represents the limit of detection. The bar represents the geometric mean of neutralization titers obtained from each group; and the error bracket represents the 95% confidence interval. Unpaired *t*-tests were performed between group pairs (see Table 1) A-B ($p < 0.05$), C-D ($p < 0.05$), C-E ($p < 0.05$), and D-E ($p < 0.05$).

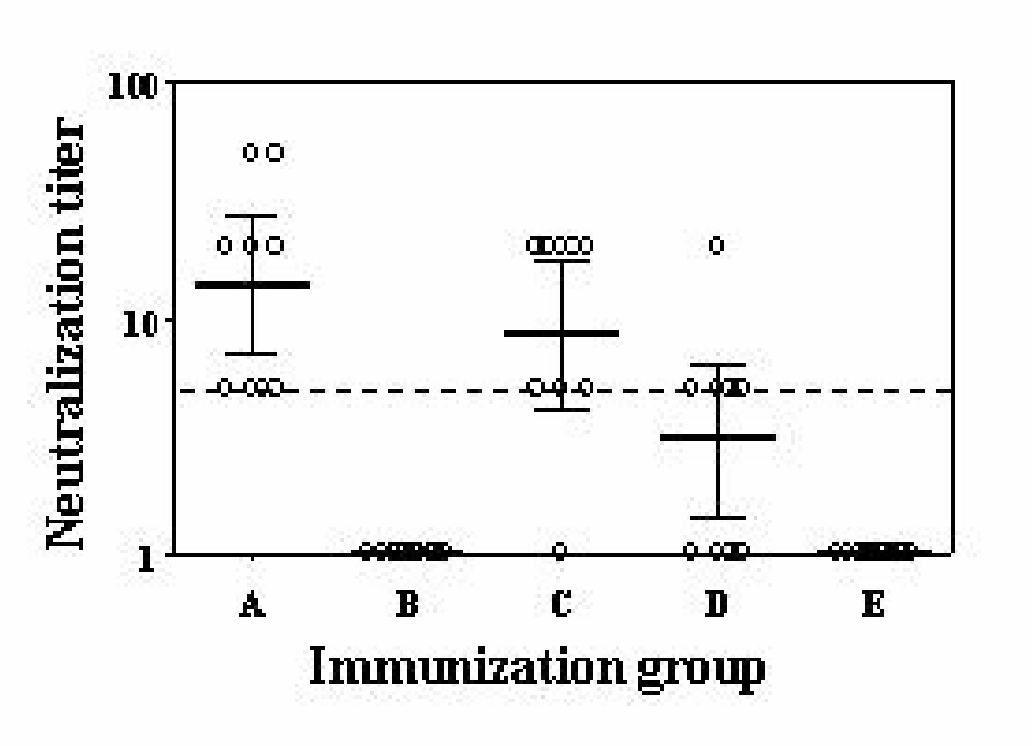
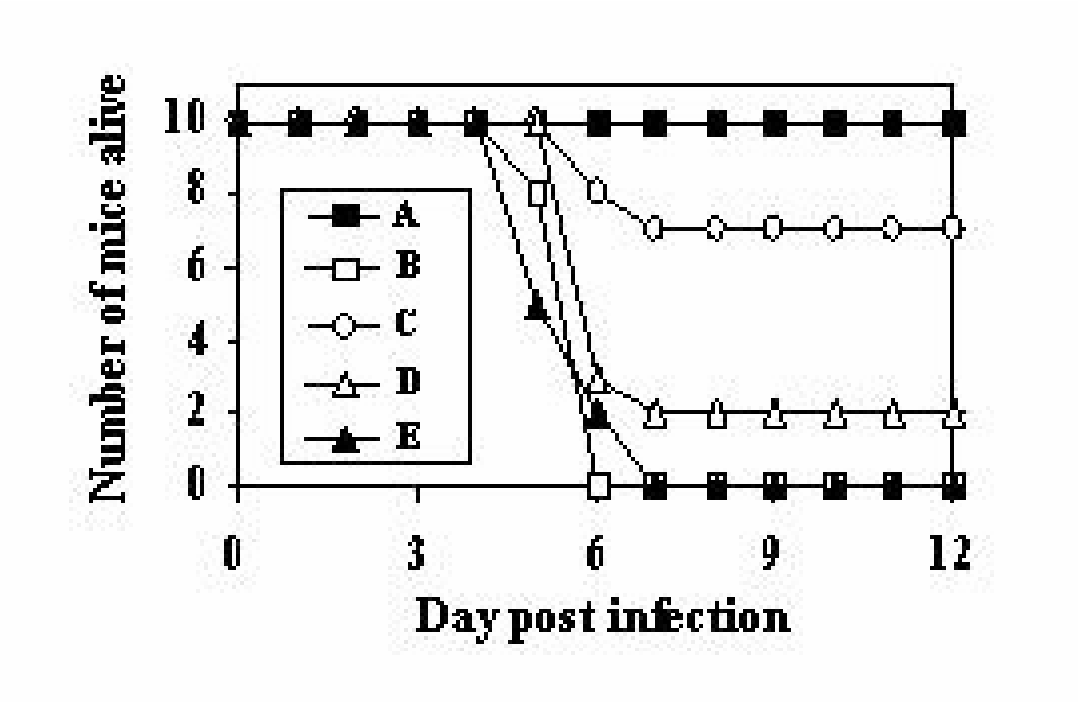

100
10
1
Neutralization titer
A
B
C
D
E
Immunization group

Figure 18. Survival of streptomycin-treated mice following systemic toxin challenge by oral infection with B2F1.

Letters in the legend box indicate immunization groups (Table 3). Statistical analysis was done on day 12 post oral infection to compare between groups A-B, C-D, C-E, and D-E using Fisher's exact test ($p < 0.05$).


Number of mice alive
10
8
6
4
2
0
A
B
C
D
E
0
3
6
9
12
Day post infection

To examine the protective efficacy of the plant-based toxoid vaccine against Shiga intoxication during an orally-acquired STEC infection, the streptomycin-treated mouse model was used (Fig. 18). In that model, infection with STEC is established through reduction of normal intestinal flora by pre-treatment of mice with streptomycin (Lindgren *et al*., 1993). In our study, mice were so treated and then fed a streptomycin-resistant isolate of *E*. *coli* serotype O91:H21 stain B2F1 that produces the highly toxic Stx2d-activatable variant Stx2. All of the mice in Immunization Group A survived challenge with B2F1. The prime boost group C was partially protected (70% survival rate), whereas only 20% of the mice in group D that received only one I.P. immunization with plant derived toxoid survived. None of the mock-immunized mice from groups B and E survived infection with B2F1. Deaths occurred between five and seven days, a pattern that is consistent with previous observations in our laboratory. Therefore, no delay in time to death was notable among the immunized mice.

Materials and Methods

Plant-optimization of Stx2 toxoid genes

The Stx2 toxoid gene was derived from pNR100 (Wen *et al*., 2005). Three additional mutations were introduced to eliminate the destabilization motifs in the $stxA_2$ region with the mutagenic primers listed in Table 4. The plant-optimized StxA2 toxoid subunit gene was amplified by PCR with primers designed to introduce 5'*Xba*I(GGAACACC TCTAGATGAAGTG) and 3'*Sac*I(GCTGAGCTCCTTTATTTA CCCG) sites flanking the $stxA_{2PO}$. The *Xba*I-*Sac*I fragment was cloned into pIBT210 (Haq *et al*., 1995) to create pNAJ65. The entire $stxB_2$ was re-synthesized by LCR (Table 4) and ligated into the *Nco*I-*Sma*I site of pBTI210.3 (Judge *et al*., 2004) to create pNAJ73. The sequence of $stxB_{2PO}$ was submitted to GenBank (DQ231140).

Construction of plant expression vectors

Two dual-expression cassettes were designed (Fig. 13). First, pNAJ65 that contained $stxA_{2PO}$ was restriction-digested to obtain a *Hin*dIII-*Sac*I fragment. Second, pNAJ73 that contained the synthetic $stxB_{2PO}$ was digested to obtain a *PstI-Eco*RI fragment. Third, pRT38 (An 1985) that contained the potato proteinase inhibitor II gene (*pin*2) was digested to obtain a *Sac*I-*Pst*I fragment. Fourth, pGPTV-Kan was digested with *Hin*dIII and *Eco*RI. All four fragments were ligated together to yield pSW3 (Fig. 13). To evaluate the effect of plant-optimization, a second dual expression cassette was

Table 4. Oligonucleotides used in this study

Primers	Sequences
Mutagenic primers used to generate *stx*$_2A_{PO}$	
253F	GTGTATATTGTTTAAATGGGTACTGTGCCTGTTACTGGG
253R	CCCAGTAACAGGCACAGTACCCATTTAAACAATATACAC
532F	CTGATTATTGAGCAAAATAATTTGTCTGTGGCCGGGTTCG
532R	CGAACCCGGCCACAGACAAATTATTTTGCTCAATAATCAG
1119F	GGCGACAGGCCTGTTATAAAAATAAGCAATACATTATGGG
1119R	CCCATAATGTATTGCTTATTTTTATAACAGGCCTGTCGCC
LCR primers used to synthesize *stx*$_2B_{PO}$	
2B1	**GCATGCATCGATCGATGCTAGCTATAGCTAGCATCGATCG**
2B2	TCAGAATCATG AAG AAG ATG TTC ATG GCT GTG CTC TTT GC
2B3	A TTG GCT TCT GTG AAT GCA TTG GCA GCT GAT TGT GCT AAG
2B4	GGT AAG ATT GAG TTC TCC AAG TACAAT GAG GAT GAT ACA T
2B5	TC ACA GTG AAG GTG GAT GGG AAG GAG TAC TGG ACC AGT AG
2B6	G TGG AAC CTC CAA CCA CTC CTC CAA AGT GCT CAA TTG ACA
2B7	GGA ATG ACT GTC ACA ATC AAG TCC AGT ACC TGT GAG TCA G
2B8	GC TCT GGA TTT GCT GAG GTG CAA TTC AAC AAT GAC TAA CC
2B9	CGGG ATGC GCAT AGTA TCAG GATC CGAT CATG ACTG CGTA
2B10	TACG CAGT CATG ATCG GATC
2B11	CTGA TACT ATGC GCAT CCCGGG TTA GTC ATT GTT GAA TTG
2B12	CAC CTC AGC AAA TCC AGA GCC TGA CTC ACA GGT ACT GGA C

2B13	TT GAT TGT GAC AGT CAT TCC TGT CAA TTG AGC ACT TTG GA
2B14	G GAG TGG TTG GAG GTT CCA CCT ACT GGT CCA GTA CTC CTT
2B15	CCC ATC CAC CTT CAC TGT GAA TGT ATC ATC CTC ATT GTA C
2B16	TT GGA GAA CTC AAT CTT ACC CTT AGC ACA ATC AGC TGC CA
2B17	A TGC ATT CAC AGA AGC CAA TGC AAA GAG CAC AGC CAT GAA
2B18	CAT CTT CTT CAT GATT CTGA CGAT CGAT GCTA GCTA TAGC
2B19	TAGC ATCG ATCG ATGC ATGC

generated (pSW2). That cassette contained *stxA*$_{2PO}$ and the native *stxB*$_2$ and was constructed in a similar fashion to pSW3.

Agrobacterium-*mediated transformation of NT-1 cells*

The method used for transformation of *Nicotiana tabacum* (NT-1) cells was originally described by An (An 1985). In brief, pSW2 or pSW3 was electroporated into *Agrobacterium tumefaciens* strain EHA105 (Hood *et al*., 1993). The resulting strains of *A*. *tumefaciens* were then used to transform NT-1 cells according to the description given by Judge *et al*. (Judge *et al*., 2004). Transformed NT-1 cells were selected on kanamycin-containing NT medium (50 μg/ml).

Immunoblot analysis

Plant-derived Stx2 toxoid expression was assessed by Western blot analysis as described previously (Judge *et al*., 2004). Rabbit anti-Stx2 polyclonal antibodies at a titer of 10,000 were used as the primary antibody. Quantitation of Stx2 toxoid produced per gram of NT-1 cells was extrapolated from a standard curve in which pixel density measured by NIH-imaging software (v. 1.61) was plotted against known concentrations of Stx2 (Sinclair *et al*., 2002).

Gb$_3$ binding assay

The Gb$_3$ binding assay, first described by Ashkenazi *et al*., was used to check assembly of the plant-derived Stx2 holotoxoid (Ashkenazi *et al*., 1989). Gb$_3$ (Matreya, Inc. State College, PA) dissolved in chloroform-methanol (2:1) was used to coat 96-well

ELISA plates (VWR International, Inc., Bridgeport, NJ) (1 μg/well). Wells were blocked with 5% (w/v) bovine serum albumin in 0.05% Tween-phosphate buffered saline (PBS), and serially diluted NT-1 or *E. coli* sonic lysates were added. The primary antibody, a monoclonal anti-Stx2, 11E10 (Melton-Celsa *et al*., 1998) , was diluted 1:1000 and a horseradish peroxidase-conjugated (HRP) goat anti-mouse IgG served as the secondary antibody (Bio-Rad Laboratories, Richmond, CA) (Perera *et al*., 1988). The antigen-antibody reaction was detected by absorbance at 600nm.

Parenteral immunization of mice

Female 3- to 4- week-old BALB/c mice (Charles River Laboratories, Wilmington, MA) in groups of 10 received I.P. injections on days 0 and 21 with 50 μl of cell extract from NT #3, NT-1Kan^R, *E. coli* that harbored pNR100, or *E. coli* that carried the pBluescribe vector alone. Toxoid concentrations were standardized to 1 μg of toxoid per immunization and the samples were mixed with an equal volume of TiterMax Gold (TiterMax USA, Inc., Norcross, GA) adjuvant for administration. Serum samples were collected before and after immunization from tail veins to monitor the production of Stx2-neutralizing serum antibodies. Mice were challenged on day 35 by I.P. injection of 20 μg Stx2 (≈10 LD_{50}).

Oral immunization and infection of mice

The immunization protocols are shown in Table 1. Female 3- to 4- week-old BALB/c mice in five groups of ten were individually fed 5 g of NT-1 cells (≈30 μg of Stx2 toxoid) mixed with 0.5 g of sucrose. The prime-boost groups first received an I.P.

injection of NT-1 cell extract that contained 1 μg of toxoid with adjuvant as above, and then were fed NT-1 cells. Serum and fecal pellets were collected before immunization and 7 days after the last dose and tested for Stx2-neutralizing serum antibody and Stx2-specific fecal IgA. On day 41 post initial immunization, all mice were fasted overnight, given streptomycin-containing water (5 g/mL), and fed 10^6 CFU of a spontaneously derived streptomycin-resistant mutant of *E. coli* strain B2F1 the next day (Wadolkowski *et al*., 1990).

Serum neutralization assay

The presence of Stx2-neutralizing antibody in serum samples was assessed by the Vero cytotoxicity neutralization assay as described previously (Wen *et al*., 2005; Gentry *et al*., 1980).

Stx2-specific fecal IgA and serum IgG ELISA

Fecal extracts were prepared in PBS and standardized by weight as previously described (Judge *et al*., 2004). ELISA plates were coated with purified native Stx2 (100 ng/well). Fecal extracts were serially diluted 10-fold, applied to the Stx2-coated plates, and incubated at 37ºC for two hours. HRP-conjugated rabbit anti-mouse IgA was used as the secondary antibody (Bio-Rad Laboratories). The antigen-antibody reaction was detected as described for the Gb_3 binding assay. Titers were defined as the reciprocal of the highest dilution of post-immune fecal extract that contained Stx2-specific antibodies as determined by optical densities greater than those seen with pre-immune fecal extract and blank wells that contained all reagents except fecal extract. Serum anti-Stx2 IgG was

also detected by ELISA as described above except that HRP-conjugated goat anti-mouse IgG was used as the secondary antibody (Bio-Rad Laboratories).

Chapter Four

Expression of EHEC intimin in transgenic maize

(work in progress)

Introduction

Intimin, as shown by both *in vitro* and *in vivo* data, is required for EHEC O157:H7 to colonize mammalian hosts (Donnenberg *et al*., 1993b; McKee *et al*., 1995). Moreover, antibodies specific to intimin can block adherence of the bacteria to both cultured cells (Gansheroff *et al*., 1999) and to the intestines of weanling piglets (an-Nystrom et al., 2002). Recently, Nicole Judge in our laboratory has developed an NT-1 cell line that expressed the C-terminal third ($C_{1/3}$), or the Tir-binding domain, of EHEC intimin. Dr. Judge then demonstrated that parenteral priming of mice with intimin purified from these transgenic NT-1 cells followed by oral feeding of the same intimin-expressing transgenic cells promoted the development of an intimin-specific mucosal immune response. This immune response appeared to correlate with a statistically significant reduction in the duration of EHEC O157:H7 colonization (as monitored by number of organisms shed in feces) upon challenged of these immunized animals with the organism (Judge *et al*., 2004). In collaboration with Dr. Wayne Curtis of Penn State University and Dr. Evelyn Dean-Nystrom of the USDA, the production of the transgenic intimin-expressing NT-1 line was scaled up for testing in cattle (unpublished data). Preliminary results showed that calves that were fed the lyophilized transgenic plant material developed an intimin-specific antibody response (unpublished data). The findings from these calf immunization studies together with the results from our mouse experiments described above, encouraged us to initiate development of an intimin-based vaccine in a more suitable plant for cattle, such as corn. Our long-range goal is to design a practical and low-cost means of immunizing cattle with intimin, and accordingly, diminish the risk of transmission of *E. coli* O157:H7 to humans.

In collaboration with Dr. Kan Wang of Iowa State University, we sought to express the C-terminal third ($C_{1/3}$) of EHEC intimin in the endosperm of transgenic maize (Fig. 19). There are several advantages, listed below, to directing the expression of a protein to this compartment of corn seed. First, the protein associates with starch and thus can be co-purified in the starch fraction. Second, corn meal has been proposed as an alternative method to orally deliver a transgenic plant vaccine (Tacket 2004). As part of the process required to make corn meal, the starch in the endosperm gelatinizes at a high temperature, Indeed, Chikwamba *et al*. who successfully expressed LT-B in the maize endosperm found that the maize kernel-derived LT-B was stable at high temperature (Chikwamba *et al*., 2003).

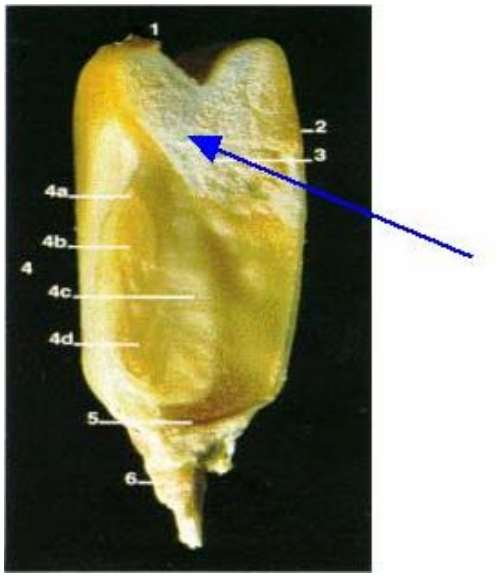
One scenario for generating transgenic maize that expresses intimin is as follows. First, the $C_{1/3}$ initimin gene is cloned under the regulation of the maize endosperm-specifc 27-kDa γ-zein promoter (Marks *et al*., 1985). Second, the *Agrobacterium*-mediated transformation method is used to introduce the intimin gene into maize cell line. Third, mature seeds from the transgenic maize plants are obtained by screening them for intimin-expression. The major drawback to this general approach is that the latter screening step cannot be done until the plants mature, since intimin, under the γ-zein promoter, would only be expressed in the endosperm of mature maize. Furthermore, the duration from the transformation stage to a mature maize plant is almost a year. For this reason, we modified this approach. Our overall scheme for preparing transgenic corn that express intimin is illustrated in (Fig. 20). Our method is a variation of the protocol above, but we opted for a quicker alternative to the second step. Here, we introduced the intimin-expressing binary vector that was created for *Agrobacterium*-mediated

transformation of transgenic NT-1(Judge *et al*., 2004) by biolistic bombardment into maize calluses. Because the intimin gene in this vector is under the ubiquitous P35 promoter, we predicted that the expression of intimin would be detectable at the callus stage.

Figure 19. Maize kernel and callus.

A: the kernel is developed during the reproductive stage of the maize life cycle. The endosperm (blue arrow), where the storage proteins are located, also harbors starch granules. **B:** This type of maize callus culture, so called type II callus, is highly embryogenic, white or pale yellow, friable, and rapidly growing. Microprojectile bombardment using DNA-coated particles has been used to transform type II calluses, which, in turn, have subsequently been regenerated into mature transgenic plants (Fromm *et al*., 1990; Gordon-Kamm *et al*., 1990). Pictures obtained from http://maize.agron.iastate.edu/corngrows.html.

A



B

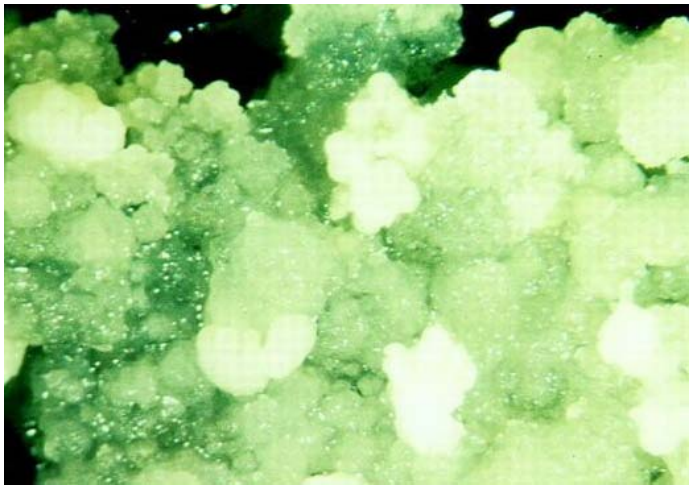
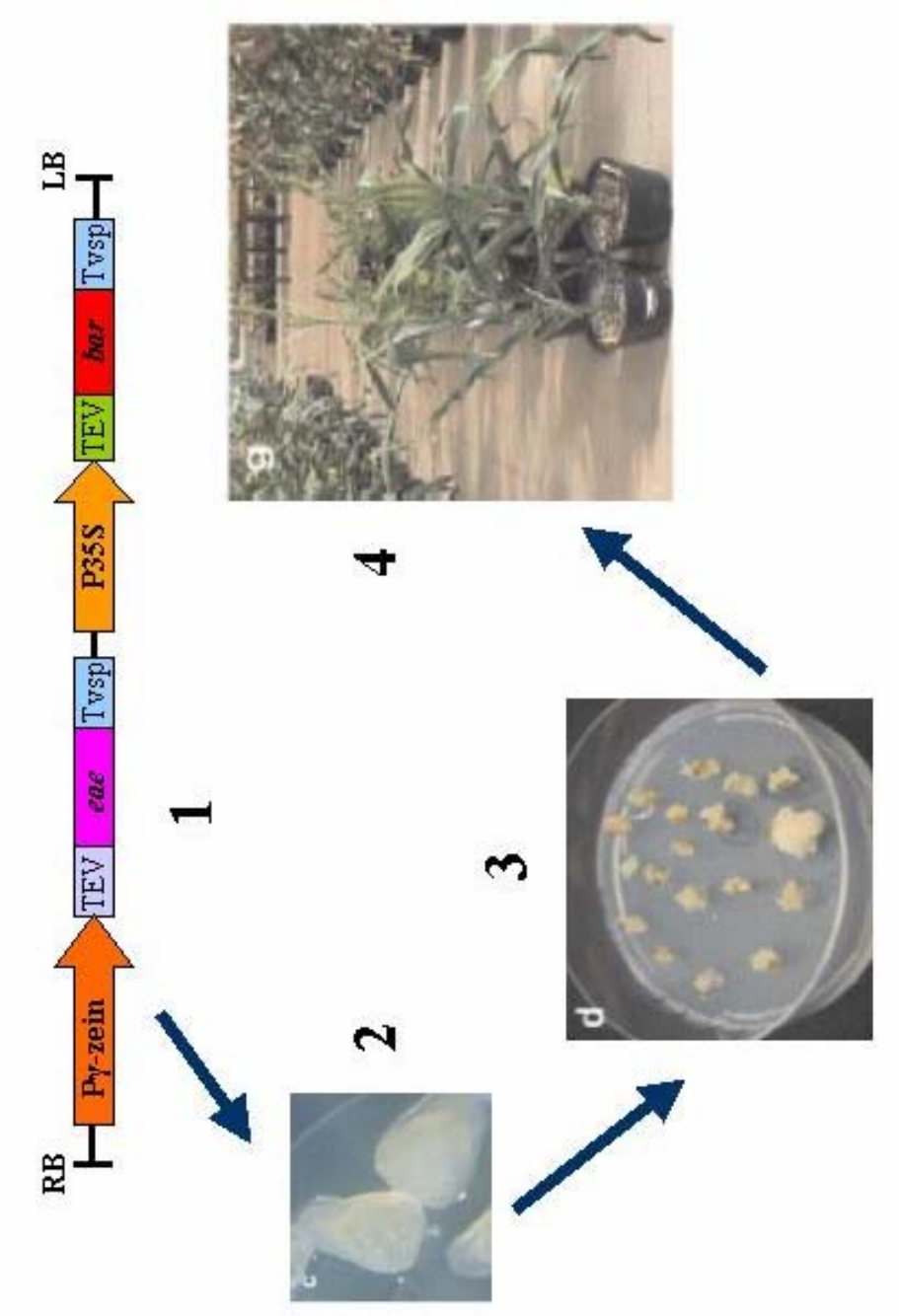


Figure 20. Overview of the development of EHEC intimin-expressing transgenic maize.

1. Use a binary vector similar to the one generated for Stx2 toxoid-expression in tobacco cell line to electroporate into *Agrobacterium*. This binary vector contain these elements between the right and left borders: the γ-zein promoter (Pγ-zein); tobacco etch virus (TEV) translational enhancer leader sequence; $C_{1/3}$ EHEC intimin gene (*eae*); soybean vegetative storage protein terminator (Tvsp); 35S Promoter (P35S); TEV enhancer; bialaphos resistance marker (*bar*); and Tvsp. **2.** Subject immature zygotic embryos dissected from corn ear to *Agrobacterium*-mediated transformation. **3.** Select for stable transformation events by growing individual callus lines on bialaphos-containing medium. **4.** Generate transformed calluses into transgenic plants, then grow them to maturity in a greenhouse.


RB
Py-zein
TEV
zae
Tvsp
P35S
TEV
bar
Tvsp
LB
1
2
3
4

Material and methods

Particle bombardment of maize callus

Plasmid DNA from pNR49 and pNR50 (Judge *et al*., 2004) was purified with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, concentrated to 1μg/μl, and then submitted to the Plant Transformation Facility in Iowa State University. The bombardment of maize calluses with this DNA was done as previously detailed by Chikwamba *et al*., 2003 and http://www.agron.iastate.edu/ptf/. Transformants were selected on bialaphos, an herbicide, containing media.

DNA extraction and PCR amplification of eae *in maize callus*

One gram of frozen, plasmid-bombarded maize callus tissue was ground with a mortar and pestle to a fine powder. Approximately 5 mg of this fine maize powder was placed in a 1.5-ml centrifuge tube, and chromosomal DNA was extracted with the DNeasy Plant Mini kit (Qiagen) according to the to the manufacturer's protocol. The final samples yielded 30-50 ng/μl of chromosomal DNA. PCR was used to amplify *eae* from the maize DNA with the primers 5'-cggatcctfatcaaaccaagg-3' and 5'-ggtaccttattctacacaaaccg-3'.

RNA extraction and RT-PCR

Frozen maize calluses were pulverized as described above, and total RNA was extracted with the RNeasy Plant Mini kit (Qiagen) according to the manufacturer's protocol . The RNA samples (at 200ng/μl) were subjected to RT-PCR using Qiagen

OneStep RT-PCR Kit (Qiagen) with the same primers described above as per the procedure detailed in the kit..

Western blot analysis

One gram of maize callus was homogenized by vortex at high speed and sonicated in 1 ml of the plant extraction buffer described by Judge *et al*. (Judge *et al*., 2004). The homogenate was subjected to SDS-PAGE, transferred onto nitrocellulose, and probed with anti-intimin polyclonal (Gansheroff *et al*., 1999) or anti-his tag monoclonal antibodies. Species-appropriate horseradish peroxidase-conjugated secondary antibodies were used (Biorad, Hercules, CA).

Results

The transformed maize lines were selected on bialaphos-containing media because the maize calluses were co-bombarded with a *bar*-containing plasmid and pNR49 or pNR50. The *bar* gene is a selectable marker that confers resistance to bialaphos. Because we only selected for bialaphos resistance, we sought to first confirm the presence of *eae* in the transformed maize lines. From the PCR results (Fig 21), we concluded that the transformation of maize with pNR49 and pNR50 was indeed successful.

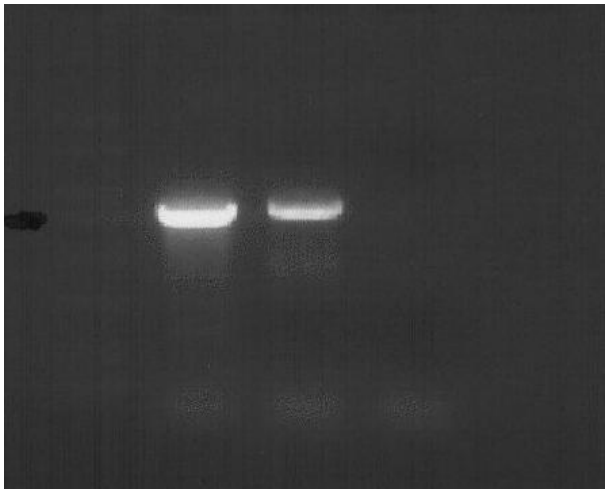
RT-PCR was used to analyze the transcription of *eae* to mRNA. We were encouraged to see that RNA samples collected from several maize lines yielded a PCR product of *eae* after incubation with reverse transcriptase (Fig. 22), though not all lines were positive. Next, we performed Western blot analysis to assess intimin expression from the maize lines that tested positive for RT-PCR of the *eae*. However, we did not detect any intimin in those lines using either anti-intimin polyclonal or anti-his tag monoclonal antibodies as probes (data not shown).

Figure 21. PCR amplification of *eae* from transformed maize lines.

A: lane 1, PCR from intimin-expressing NT-1 cells as the positive control; lane 2, PCR from a transformed maize line; lane 3, PCR from an untransformed maize line as the negative control. **B:** ten maize lines that screened positive for *eae*.

A

1 2 3



B

1 2 3 4 5 6 7 8 9 10

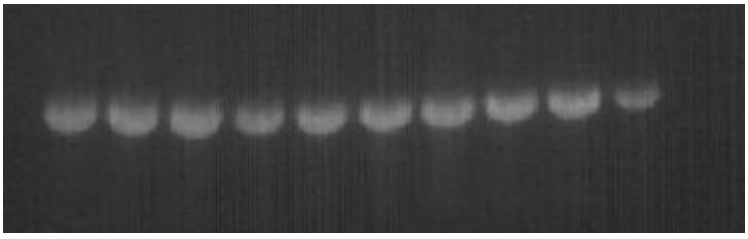
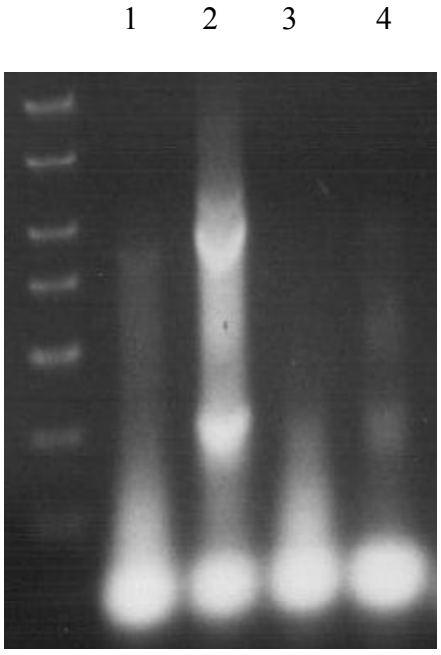


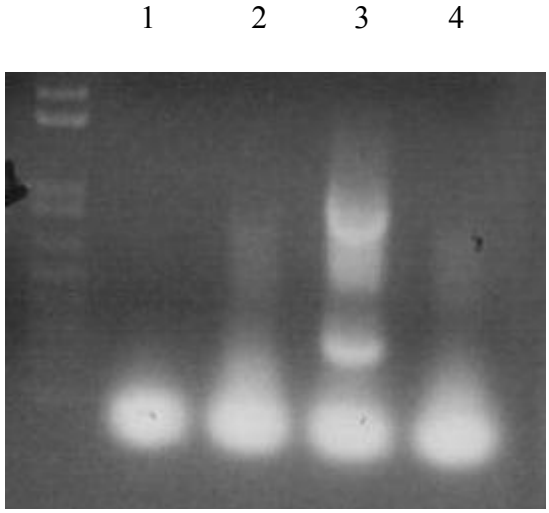
Figure 22. RT-PCR from transgenic maize.

Each RNA sample was subjected to reverse transcription with or without RT, then PCR amplified for *eae* product. **A:** lane 1, maize line#1 –RT; lane 2, maize line#1 +RT; lane 3, maize line #2 –RT; lane 4, maize line#2 +RT. **B:** lane 1, maize line #15 +RT; lane 2, maize line #15 –RT; lane 3, maize line #16 +RT; lane 4, maize line #16 –RT.

A



B



Chapter Five

Discussion and Future Directions

Overview of the results in the context of the dissertation objective and specific aims

The objective of this thesis was to further vaccine development goals against EHEC O157:H7. To address the first aim, a genetic toxoid approach was used to assess the cross-reactivity between the heterologous toxin types Stx1 and Stx2. The single toxoid-immunized mice were protected against homologous but not dual-toxin challenge, results that indicated that each toxoid conferred protection only against the homologous type. Furthermore, the analysis of the immune responses per toxoid immunization showed no cross reactivity of antisera to heterologous toxins. The data from this aim confirmed the notion that a multivalent toxoid vaccine is required to protect against strains of O157:H7 that produced both types of Stx. The second aim continued the toxoid approach by incorporating the Stx2 toxoid into a plant cell-based vaccine. The toxoid gene was first plant-optimized and then cloned into a plant-expression cassette that allowed for the co-expression of the A and B subunits. The plant-derived toxoid was characterized for its expression, folding, and immunogenicity. Lastly, the transgenic plant cells were fed to mice as a mean of oral immunization against Stx2. The survival of these animals after B2F1 oral infection challenge suggested that a plant-based oral vaccine is an effective approach for generating protection against systemic intoxication by Stx2. Finally, in the third aim, we initiated studies as part of a multi-year effort to express EHEC-intimin in transgenic maize. Ultimately, we intend to use such a transgenic plant as an oral vaccine in order to reduce the colonization "ability" of O157:H7 in cattle. The bombardment of maize calluses with the intimin-expressing tobacco vector successfully introduced DNA into those cells and our RT-PCR data showed that *eae* transcripts formed. However, our

Western blot analysis did not indicate that the transformed maize cells expressed intimin. Taken with the positive RT-PCR results, we concluded that the inability to detect intimin occurred as a consequence of some post-transcriptional problem. The remainder of this section discusses the dissertation results in more detail and concludes with a discussion of future directions derived from the findings presented here.

Genetic toxoids of Stx1 and Stx2

In this study, genetic toxoids of Stx1 and Stx2 were constructed and purified by a simple affinity chromatography method. Immunization of mice with these toxoids of Stx1 and Stx2 elicited serum IgG to the homologous toxin type but not the heterologous toxin type, and these antibodies provided toxin-specific immunity to toxin challenge. Antibodies against Stx1B were observed by Western blot in the sera of mice immunized with the Stx1 toxoid. However, the Stx2 toxoid-immunized mice did not produce detectable anti-Stx2B, even though Stx2B was present in the vaccine preparations. We concluded from this finding that Stx1B is more immunogenic than Stx2B. This conclusion is consistent with observations of Marcato *et al*., whereas Stx1B subunit vaccines have been more successful (Boyd *et al*., 1991; Acheson *et al*., 1996a). The lack of detectable Stx2B-specific antibodies on Western blot was consistent with the previously-reported findings of Padhye *et al*. (Padhye *et al*., 1989). In that study, mice were immunized with heat-treated Stx1 or Stx2. Antibodies from the Stx1-immunized mice reacted to the A, A_1 and B subunits of Stx1 on Western blot while antibodies from the Stx2-immunized mice reacted only to the A and A_1 polypeptides.

Although the toxoid preparations were free of excessive protein contaminants, endotoxin was detectable in the toxoids used to immunize mice. This finding was not surprising because the toxoids were expressed from recombinant genes in *E. coli*. Whether the amount of endotoxin in the toxoid samples was sufficient to have potentiated the immune response to the toxoids was not determined, but a recent report suggests that endotoxin can affect the immune toxin in *in vitro* assays (Westerholt *et al*., 2003). To

advance the toxoid vaccine approach to the level of human use, more precise measures, such as reduction of endotoxin by passage of the toxoid preparations through an endotoxin-binding column will be required. In addition to reduction of endotoxin contamination, the determination of effective dosages and safe adjuvants are also important issues. Of note, a recent report suggests that Shiga toxin itself possesses adjuvant activity (Ohmura *et al*., 2005), an observation that may lead to the development of a holotoxoid vaccine without the requirement for a separate adjuvant.

Lack of cross-reactivity between Stx1 and Stx2

In this investigation, we observed that rabbit polyclonal antisera reacted to Stx2B on Western blots but mouse antisera did not. This discrepancy could be due to the difference in host genetic backgrounds that resulted in MHC-restriction of antigen presentation. Another explanation is that the rabbits were immunized with formalin-treated toxin, whereas the mice in the present study were immunized with genetic toxoids. The chemical treatment may have denatured in such a way as to expose additional epitopes in the B subunit; whereas the genetic toxoids remained structurally identical to the wild-type toxin. Alternatively, the rabbit sera could merely have been of higher titer and contained a larger pool of anti-Stx2B antibodies that reacted more strongly on Western blots than did the mouse-derived antisera.

In contrast to previous studies in rabbits, neither cross-neutralization nor cross-protection was observed with these genetic toxoid immunizations when given to mice. It is not clear whether these discrepant results reflect species-specific factors in response to the toxoid molecules or whether the chemical toxoid adminstered to rabbits contained conformationally altered toxins, and engendered cross-neutralizing antibodies. In our study, the genetic toxoids induced a strong but toxin-specific protective response. Since wild-type *E. coli* O157:H7 and other strains of EHEC commonly produce both Stx1 and Stx2, we designed the dual-toxin challenge of this experiment to mimic infections with multi-toxin producing strains. A rigorous challenge dose of $50LD_{50}$ of each toxin was selected to demonstrate protection in mice to toxins in gross excess of levels expected to cause disease or to be present during the disease process. The result of

the challenge indicated that a multivalent vaccine that includes both types of toxoids is needed to confer full protection to mice against EHEC stains that produce both types of toxins. It may also be argued that a multivalent vaccine is not necessary because Stx2 is more lethal than Stx1 in mice (Tesh *et al*., 1993) and more likely to cause HUS in humans (Kleanthous *et al*., 1990). However, given the severity of the disease, and lack of cross-protection between antisera, we maintain that it is prudent to vaccinate against both serotypes of Shiga toxins so as to prevent HUS.

Development of the plant-based Stx2 vaccine

Our strategy for development of a safe effective anti-Stx2 vaccine was to start with a genetically inactivated Stx2 A subunit and to preserve the holotoxin structure by inclusion of the B subunit to maximize the epitopes available for immunization. Previous work has shown that the StxA2 subunit elicits protective antibody production in animals, whereas the StxB2 subunit alone does not (Padhye *et al*., 1989; Marcato *et al*., 2001). This is probably attributable to the requirement for the A_2 portion (N-terminus) of the StxA2 subunit for pentamerization and proper presentation of the StxB2 binding domain (Haddad *et al*., 1993). In addition, we suspect that StxA2 is immunodominant to StxB2 from our recent observation that anti-StxB2 antibodies are not detected in mice that are immunized parenterally with Stx2 holotoxoid, yet they are protected by Stx2 toxin challenge (Wen *et al*., 2005). Nonetheless, because of the important role of the StxB2 pentamer in binding Gb_3 on susceptible cells, we chose to incorporate both the A toxoid and B subunits into the plant-based vaccine.

The expression of Shiga AB_5 holotoxins in bacteria is achieved through transcription of a single mRNA that encodes the *stxA* and *stxB* genes, followed by translation initiated at two separate ribosomal binding sites (RBS). The RBS at which translation of the B subunit begins is stronger than the A subunit RBS and this accounts for the synthesis of more B than A proteins from the Stx transcript. To express the Stx2 toxoid in plants, two distinct open reading frames (each with the CaMV 35S promoter) were constructed, but no provision was made for enhanced expression of the B subunit. Western blot analysis showed that both subunits were produced in detectable amounts in NT-1 cells, but

proportionally more StxA2 was expressed than StxB2. Because the A subunit is a larger protein, it may have provided more antigenic epitopes recognizable by polyclonal antibodies than did the B subunit. It is also likely that the B subunit was not expressed in excess of A as seen in bacteria since the dual expression cassettes used the same strong promoter. In NT-1 cells, a protein of the expected size of StxA2 was detected, but a larger band of equal intensity was also seen by Western blot. We suspected that StxA2 was modified by glycosylation, as was observed when the bacterial protein intimin was expressed in NT-1 cells (Judge *et al*., 2004). An examination of the *stxA*$_2$ sequence revealed three potential Asn-linked glycosylation sites at eukaryotic "N-X-S/T" motifs. Furthermore, enzymatic deglycosylation specific for the N- and O-linked sugar residues reduced the size of the larger A subunit protein bands (data not shown). Another explanation for this observed larger StxA2 protein is that the bacterial signal peptidase cleavage site in the A subunit was not consistently recognized and cleaved in eukaryotic cells as suggested by Yu *et al*. (Yu *et al*., 2005). Nonetheless, the toxoid structure was immunogenic and provided systemic protection against Stx2-producing bacteria.

Though it is not possible to assure the native conformation of plant-derived toxoid without direct evidence from crystallography, we demonstrated association of the plant-derived toxoid A and B subunits with a Gb_3-based ELISA. Less plant-derived holotoxoid was detected by Gb_3 ELISA than was bacterially-derived toxoid. This reduction may be indicative of less efficient assembly of plant-derived holotoxoid because subunits were not synthesized in the optimal 1A:5B ratio. Another explanation is that detection of holotoxoid with anti-StxA2 antibody was reduced due to the glycosylation of the A subunit.

Immunization in mice using the plant vaccine

To test the immunogenicity of the plant-derived toxoid, we first administered plant extracts intraperitoneally to mice and found that they were protected against lethal toxin challenge. Then, the transformed plant material was given orally over several feedings and protection against challenge with a lethal oral infection of Shiga toxin-producing *E. coli* was achieved. In contrast to other work (Kong *et al*., 2001), the prime-boost approach of combined parenteral and oral immunization did not seem to have a benefit in this study. In fact, the prime-boost method was shown to be less protective than the feeding method alone. However, the parenteral priming was effective by comparison between the prime-mock boost group (D) and the negative control group (E). Similarly, the two oral boosts were effective as shown by the comparison between groups C and D. Taken together, the sub-optimal level of protection afforded by the prime-boost method probably reflects the fact that the prime-boost group received fewer boosts overall than the orally vaccinated mice. Others have shown that prime-boost strategy in reverse order (oral priming followed by I.P. boosts) has worked well to elicit systemic immunity (Devico *et al*., 2002). However, multiple parenteral boosts would defeat one of our initial goals in development of a plant-based vaccine, which was to minimize the use of needles.

Some mice in the mock-immunized groups (B and E) exhibited fecal antibodies that were reactive by ELISA. This raises an important consideration for the evaluation of orally-administered vaccines in general. The gut provides an effective but very non-selective path by which to introduce antigens. Production of fecal antibodies may indicate sensitization to the immunizing antigen or a non-specific reaction to previously

encountered related antigens. Here, fecal IgA titers did not necessarily predict the development of a protective response against the systemic effects of Stx2. In contrast, the Vero cytotoxicity neutralization assay, a functional assay done with sera that represented a broader antibody class response was far more indicative of *in vivo* protection.

Although subunits of other bacterial toxins of AB_5 family, such as heat labile toxin (LT) of enterotoxingenic *E. coli* and cholera toxin (CT) of *Vibrio cholerae* have been expressed in plant systems for vaccine purposes (Mason *et al*., 1998; Arakawa *et al*., 1997), the expression of an AB_5 holotoxoid of bacterial origin in the plant system is unprecedented. In contrast to LT and CT that act locally at the gastrointestinal level where mucosal immunization is most likely to be effective, Stxs enter the circulation and act systemically to target tissues that express the Gb_3; thus, systemic immunity is required to protect against the hemolytic uremic syndrome. The streptomycin-treated mouse model of STEC infection is not completely representative of human STEC infection since mice do not experience diarrhea and they suffer renal tubular damage as opposed to the glomerular necrosis seen in humans. Nonetheless, the prevention of deaths, the usual outcome of such an infection in mice, serves as proof-of-concept that oral immunization with Stx2 toxoid administered via plant-based vaccine is protective against systemic toxin-mediated disease. Additional steps are needed to prepare Stx2 holotoxoid for use as an oral plant-based vaccine for humans. A more suitable host plant, such as tomatoes or bananas, would be desirable and different promoters would likely be required for optimal expression in other fruits or vegetables. In addition to fresh plant-based vaccines, dried or lyophilized plant materials also provide a good vaccine vehicle in

which antigens are stably preserved and concentrated (Tacket 2004). By reducing the mass of the plant vaccine, such as encapsulating the material into pill form, oral intake would be easier and uniform doses would be better controlled. Moreover, the inclusion of a similar Stx1 toxoid would be necessary to provide protection against Stx- and Stx1-producing organisms such as *Shigella dysenteriae* type 1 and many other Shiga toxin-producing *E. coli*, respectively. A composite approach to plant vaccines against several diarrheal agents would be ideal. We feel that the systemic immunity against Stx2 that we observed indicates that an oral plant-based vaccine shows promise for protection against the severe life-threatening consequences of Shiga toxin-producing *E. coli* infection against which there are no other suitable treatments or therapies currently available.

Expression of intimin in transgenic maize

Several explanations can be offered for the failure to detect intimin expression in transgenic maize calluses. First, since intimin mRNA was detected, the absence of intimin protein suggests a problem with translation. However, in eukaryotic cells, transcription and translation are not coupled; therefore the mRNA may have been degraded before translation could be completed. Second, the codon usage in maize is certainly different from that of *E. coli*, and may need to optimize the codon in *eae* for maize expression. Third, the sensitivity of the Western blot assay may have been insufficient to detect low levels of intimin expression in maize. Fourth, the intimin expressed in maize may not be soluble or stable, and therefore, not available for recognition by antibodies. To test this, purified, bacterially-derived intimin was added into untranformed maize callus extract and subjected to SDS-PAGE and Western blot with anti-intimin polyclonal antibodies. The antibody recognized the antigen, which suggests the protein is stable in the plant extract. Despite the failure to detect intimin, we remain optimistic because failure to detect a protein in the bombarded maize calluses does not always equate to failure to find the protein in transgenic maize plants (Dr. Kan Wang, Iowa State University, personal communication).

Future directions

The data from the toxoid studies confirmed the need for a multivalent vaccine that protects against both types of Stx. A hybrid toxoid that contains protective epitopes from both Stx1 and Stx2 could satisfy such a requirement. Currently, our laboratory has developed a hybrid toxoid that consists of Stx2 A subunit and Stx1 B subunit. Further testing is underway to characterize that toxoid, i.e. holotoxin conformation, protective antigenicity.

Since this thesis focuses on oral vaccines, one future goal is to construct a plant-based multivalent vaccine for Stx1, Stx2, and EHEC and EPEC intimin for use in humans. One of the advantages of using plant-based vaccine is the flexibility to include more than one antigen. As demonstrated with the Stx2 toxoid expressing-transgenic tobacco cells, multiple expressing cassettes can be incorporated into the *Agrobacterium* transformation vector. Alternatively, individual plant cell lines could be developed to express different antigens and combined with oral feeding as a mean of immunization with a multivalent vaccine.

The use of tobacco cells in our study was a proof-of-concept study. Once we have established the protective efficacy of the plant-based oral vaccines, the next step would be to express the antigen in a different plant system that is more suitable for clinical trials, such as potato or maize. The progress in intimin expression in maize has been challenging yet encouraging. The next step is to codon-optimize *eae* for maize expression. Then the optimized *eae* will be cloned under the regulation of γ-zein

promoter for expression in the endosperm. We firmly believe such an EHEC intimin-oral vaccine would reduce the transmission of O157:H7 from ruminant animals to human.

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